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CUTINASE VARIANTS

FIELD OF THE INVENTION

The present invention relates to a cutinase variant, more particularly to a cutinase variant having improved thermostability. The invention also relates to a DNA sequence encoding the variant, a vector comprising the DNA sequence, a transformed host cell harboring the DNA sequence or the vector, to a method of producing the variant, and to use of the variant.

BACKGROUND OF THE INVENTION

Cutinases are lipolytic enzymes capable of hydrolyzing the substrate cutin. Cutinases are known from various fungi (P.E. Kolattukudy in "Lipases", Ed. B. Borgström and H.L. Brockman, Elsevier 1984, 471-504). The amino acid sequence and the crystal structure of a cutinase of *Fusarium solani pisi* have been described (S. Longhi et al., Journal of Molecular Biology, 268 (4), 779-799 (1997)). The amino acid sequence of a cutinase from *Humicola insolens* has also been published (US 5,827,719).

A number of variants of the cutinase of *Fusarium solani pisi* have been published: WO 94/14963; WO 94/14964; Appl. Environm. Microbiol. 64, 2794-2799, 1998; Proteins: Structure, Function and Genetics 26, 442-458, 1996; J. of Computational Chemistry 17, 1783-1803, 1996; Protein Engineering 6, 157-165, 1993; Proteins: Structure, Function, and Genetics 33, 253-264, 1998.

Fungal cutinases may be used in the enzymatic hydrolysis of cyclic oligomers of poly(ethylene terephthalate), e.g. in the finishing of yarn or fabric from poly(ethylene terephthalate) fibers (WO 97/27237). However, it is desirable to improve the thermostability of known fungal cutinases to allow a higher process temperature.

SUMMARY OF THE INVENTION

The inventors have found certain variants of fungal cutinases having improved thermostability.

Accordingly, the invention provides a variant of a parent fungal cutinase comprising substitution of one or more amino acid residues which is located:

a) within 17 Å from the location of the N-terminal amino acid (as calculated from amino acid residues in a crystal structure), and/or

b) within 20 positions from the N-terminal amino acid.

The invention also provides a DNA sequence encoding the variant, an expression vector comprising the DNA sequence, a transformed host cell harboring the DNA sequence or the expression vector, and a method of producing the variant by cultivating the transformed host cell so as to produce the variant and recovering the variant from the resulting broth. The invention also provides a process for enzymatic hydrolysis of cyclic oligomers of poly(ethylene terephthalate) by treatment with the cutinase variant and a detergent composition comprising the variant.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the 3D structure of the cutinase of *H. insolens*.

Fig. 2 is a computer model showing the three-dimensional structures of the cutinases from *F. solani pisi* (left) and *H. insolens* (right). Different colors have been used to identify the N-terminal amino acid and zones of 12 Å and 17 Å diameter around this.

Figs. 3-6 illustrate the hydrolysis of c3ET. Details are given in the Examples.

DETAILED DESCRIPTION OF THE INVENTION

Fungal cutinase

The parent cutinase is a fungal cutinase, particularly a filamentous fungal cutinase, preferably native to a strain of *Humicola* or *Fusarium*, more preferably *H. insolens* or *F. solani pisi*, most preferably *H. insolens* strain DSM 1800.

The amino acid sequence of the cutinase of *H. insolens* strain DSM 1800 and the DNA sequence encoding it are shown as SEQ ID NO: 2 and SEQ ID NO: 1 of US 5,827,719. The numbering system used herein for the *H. insolens* cutinase is based on the mature peptide, as shown in said SEQ ID NO: 2.

The amino acid sequence of the cutinase of *F. solani pisi* is shown as the mature peptide in Fig. 1D of WO 94/14964. The numbering system used herein for the *F. solani pisi* cutinase is that used in WO 94/14964; it includes the pro-sequence shown in said Fig. 1D; thus, the mature cutinase is at positions 16-214.

The parent cutinase preferably has an amino acid sequence which is at least 50 % (particularly at least 70 % or at least 80 %) homologous to the cutinase of *H. insolens* strain DSM 1800. Preferably, the parent cutinase is one that can be aligned with the cutinase of *H. insolens* strain DSM 1800.

Homology and alignment

For purposes of the present invention, the degree of homology may be suitably determined according to the method described in Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45, with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1. The determination may be done by means of a computer program known such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711).

Two given sequences can be aligned according to the method described in Needleman (*supra*) using the same parameters. This may be done by means of the GAP program (*supra*)..

Three-dimensional structure of cutinase

The structure of the cutinase of *H. insolens* was solved in accordance with the principle for X-ray crystallographic methods as given, for example, in X-Ray Structure Determination, Stout, G.K. and Jensen, L.H., John Wiley & Sons, Inc. NY, 1989. The structural coordinates for the solved crystal structure at 2.2 Å resolution using the isomorphous replacement method are given in Fig. 1 in standard PDB format (Protein Data Bank, Brookhaven National Laboratory, Brookhaven, CT).

The structure of the cutinase of *F. solani pisi* is described in Martinez et al. (1992) Nature 356, 615-618. The 3D structures of the cutinases of *F. solani pisi* and *H. insolens* are compared as a computer model in Fig. 2.

It should be noted that the overall three-dimensional structure of the fungal cutinases is very similar and has been shown by X-ray crystallography to be highly homologous. The similarities between the cutinases from *F. solani pisi* and *H. insolens* is clearly apparent from the computer model in Fig. 2. It is therefore to be expected that modifications of the type indicated for one fungal cutinase will also be functional for other fungal cutinases.

Substitution near N-terminal

The variant of the invention has one or more amino acid substitutions in the vicinity of the N-terminal. The substitution is within a distance of 17 Å (preferably within 12 Å) and/or within 20 positions (preferably within 15 positions) of the N-terminal. The distance from the N-terminal is to be calculated between the Cα atom of the amino acids, and is calculated from an amino acid in a crystal structure (i.e. visible in the X-ray structure).

In the cutinase of *H. insolens* strain DSM 1800, the two N-terminal amino acids Q1 and L2 are not visible in the X-ray structure, so the distance is to be calculated from amino acid G3. Amino acids within 17 Å include positions 3-12, 18, 20-60, 62-64, 82, 85-86, 100-108, 110-111, 130-132, 174, 176-182, 184-185, 188, and 192. Those within 12 Å include positions 3-8, 22-27, 30-47, 53-59, 102, 177, and 180-181.

In the cutinase of *F. solani pisi*, the N-terminal amino acid G17 is visible in the X-ray structure. Amino acids within 17 Å include positions 17-26, 34-75, 77-79, 101, 115, 117-119, 147, 191-197, 199-200, and 203. Those within 12 Å include positions 17-22, 38, 40, 45-58, 60, 65, and 70-72.

The variants of the invention have improved thermostability compared to the parent enzyme. The thermostability may be determined from the denaturation temperature by DSC (differential scanning calorimetry), e.g. as described in an example, e.g. at pH 8.5 with a scan rate of 90 K/hr. Preferred variants have a denaturation temperature which is at least 5°C higher than the parent enzyme.

The total number of substitutions in the above regions is typically not more than 10, e.g. not more than substitutions in the above regions. In addition, the cutinase variant of the invention may optionally include other modifications of the parent enzyme, typically not more than 10, e.g. not more than 5 alterations (substitutions, deletions or insertions) outside of the above regions. Thus, the total amino acid sequence of the variant may have not more than 20, e.g. not more than 10 alterations compared to the parent cutinase.

Solvent accessible surface

One or more of the substitutions is preferably made at an exposed amino acid residue, i.e. an amino acid residue having a solvent accessible surface. This can be calculated by teh "dssp" program (version October 1988) described in W. Kabsch and C. Sander, Biopolymers, 22 (1983) pp. 2577-2637.

In the cutinase of *H. insolens* strain DSM 1800, the following amino acids lie within 17 Å of G3 at the N-terminal and have a solvent accessible surface greater than 0: 3-12, 18, 26-33, 36-38, 40-45, 47-56, 59-60, 62-64, 82, 85-86, 104-105, 174, 176-179, 181-182, 192.

Preferred substitutions

A preferred substitution near the N-terminal is one that increases the electrical charge, i.e. a substitution of a negatively charged amino acid with a neutral or positively charged amino acid or substitution of a neutral amino acid with a positively charged amino acid. This substitution may be made at a position corresponding to position E6, E10, E47 or E179 in the cutinase of *Humicola insolens* strain DSM 1800, preferably a substitution corresponding to E6Q, E10Q, E47K or E179Q.

Another preferred substitution near the N-terminal is substitution with a Pro residue, preferably a substitution corresponding to A14P or R51P in the cutinase of *Humicola insolens* strain DSM 1800.

Preferred variants

The following are some preferred variants in the *H. insolens* cutinase. Corresponding variants of other parent cutinases are also preferred. (JC numbers are the inventors' designations).

JC006: R51P

JC011: E6Q, L138I

JC013: A14P, E47K

JC014, JC015: E47K

JC025: E179Q

JC026: E6Q, E47K, R51P

JC029: A14P, E47K, E179Q

JC030: E47K, E179Q

JC031: E47K, D63N

JC038: E6Q, A14P, E47K, R51P, E179Q

JC039: E6Q, E10Q, A14P, E47K, R51P, E179Q

JC040: Q1P, L2V, S11C, N15T, F24Y, L46I, E47K

Use of cutinase variant

The cutinase variant of the invention may be used, e.g., for the enzymatic hydrolysis of cyclic oligomers of poly(ethylene terephthalate), such as cyclic tri(ethylene terephthalate), abbreviated as c3ET.

In particular, this may be used to remove such cyclic oligomers from polyester containing fabric or yarn by treating the fabric or yarn with the cutinase variant, preferably followed by rinsing the fabric or yarn with an aqueous solution having a pH in the range of from about pH 7 to about pH 11. The treatment of polyester is preferably carried out above the glass transition temperature of c3ET (about 55°C) and below the glass transition temperature of polyester (about 70°C). Thus, the treatment is preferably carried out at 55-70°C, e.g. at 60-65°C. The process may be carried out in analogy with WO 97/27237.

The cutinase variant of the invention is also useful in detergents, where it may be incorporated to improve the removal of fatty soiling, as described in WO 94/03578 and WO 94/14964.

Nomenclature for amino acid alterations

The nomenclature used herein for defining mutations is basically as described in WO 92/05249. Thus, R51P indicates substitution of R in position 51 with P.

Methods for preparing cutinase variants

The cutinase variant of the invention can be prepared by methods known in the art, e.g. as described in WO 94/14963 or WO 94/14964 (Unilever). The following describes methods for the cloning of cutinase-encoding DNA sequences, followed by methods for generating mutations at specific sites within the cutinase-encoding sequence.

Cloning a DNA sequence encoding a cutinase

The DNA sequence encoding a parent cutinase may be isolated from any cell or microorganism producing the cutinase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the cutinase to be studied. Then, if the amino acid sequence of the cutinase is known, labeled oligonucleotide probes may be synthesized and used to identify cutinase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labeled oligonucleotide probe containing sequences homologous to another known cutinase gene could be used as a probe to identify cutinase-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying cutinase-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming cutinase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for cutinase (*i.e.* maltose), thereby allowing clones expressing the cutinase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, *e.g.* the phosphoroamidite method described S.L. Beaucage and M.H. Caruthers, (1981), Tetrahedron Letters 22, p. 1859-1869, or the method described by Matthes et al., (1984), EMBO J. 3, p. 801-805. In the phosphoroamidite method, oligonucleotides are synthesized, *e.g.* in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al., (1988), Science 239, 1988, pp. 487-491.

Site-directed mutagenesis

Once a cutinase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites. In a specific method, a single-stranded gap of DNA, the cutinase-encoding sequence, is created in a vector carrying the cutinase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al., (1984), Biotechnology 2, p. 646-639. US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method for introducing mutations into cutinase-encoding DNA sequences is described in Nelson and Long, (1989), Analytical Biochemistry 180, p. 147-151. It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Expression of cutinase variants

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

Expression vector

The recombinant expression vector carrying the DNA sequence encoding a cutinase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. The vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. Examples of suitable expression vectors include pMT838.

Promoter

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the DNA sequence encoding a cutinase variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, the TPI (triose phosphate isomerase) promoter from *S. cerevisiae* (Alber et al. (1982), J. Mol. Appl. Genet 1, p. 419-434, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

Expression vector

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α-amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, *e.g.* as described in WO 91/17243.

The procedures used to ligate the DNA construct of the invention encoding a cutinase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

Host Cells

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a cutinase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, *e.g.* by homendocina strain SD 702, which was then inoculated on a plate medium containing the lipase substrate to select a strain without any clear zone formed, herein, the strain LD 9 was recovered.

iii) Bacillus

An oligonucleotide capable of adding the XbaI site to the N-terminus of mature lipase, as shown as Sequence No.10, was chemically synthesized. Additionally, an oligonucleotide capable of adding the XbaI site to the 3'-terminus of the gene encoding the protein involved in the production of the lipase, as shown as Sequence No.11, was chemically synthesized. Mixing these two oligonucleotides with a plasmid pS1E for polymerase chain reaction (PCR), a DNA fragment carrying the mature lipase gene and the gene encoding the protein involved in the production thereof was recovered. After completely digesting the fragment with XbaI and linking the digested fragments with plasmid pUC118, a plasmid pSM1 was recovered, on which a DNA fragment carrying the mature lipase gene and the gene encoding the protein involved in the production thereof was harbored and inserted in the XbaI digested site of pUC118.

An oligonucleotide capable of adding the XbaI site to the C-terminus of the lipase, as shown as Sequence No.12, was chemically synthesized. Mixing the oligonucleotide and the oligonucleotide as shown as Sequence No.10 with the plasmid pS1E for polymerase chain reaction (PCR), a DNA fragment carrying only the mature lipase gene was recovered. After completely digesting the fragment with XbaI and linking the digested fragments with the plasmid pUC 118 in the same manner as described above, a plasmid pSM2 was recovered, on which a DNA fragment carrying the mature lipase gene and the gene encoding the protein involved in the production thereof was harbored and inserted in the XbaI digested site of pUC118.

Oligonucleotides as shown as Sequence Nos. 13 and 14 were chemically synthesized. Mixing these two oligonucleotides with plasmid pSDT812 (Japanese Patent Laid-open No. Hei 1-141596) for polymerase chain reaction (PCR), a DNA fragment including the promoter region of alkali protease and a part of the prepro sequence thereof was recovered. After completely digesting the fragment with EcoRI and XbaI and linking the digested fragments with plasmid pUC118, a plasmid pAP1 was recovered, on which the DNA fragment including the promoter region of alkali protease and a part of the prepro sequence thereof was harbored and inserted in between the EcoRI digestion site and the XbaI digestion site of pUC118.

Oligonucleotides as shown as Sequence Nos.15 and 16 were chemically synthesized. Mixing these oligonucleotides with the plasmid pSDT812 for polymerase chain reaction (PCR), a DNA fragment including the terminator region of alkali protease was recovered. After completely digesting the fragment with XbaI and HindIII and linking the digested fragments with a plasmid pUC 118 in the same manner as described above, a plasmid pAP2 was recovered, on which a DNA fragment including the terminator region of alkali protease was harbored and inserted in between the XbaI digested site and HindIII digested site of pUC118.

Digesting the plasmid pAP1 with EcoRI and XbaI, a DNA fragment including the promoter region of alkali protease and a part of the prepro sequence thereof was recovered. Additionally, digesting the plasmid pAP2 with XbaI and HindIII, a DNA fragment including the terminator region of alkali protease was recovered. Linking then these two fragments to the plasmid pUC118, a plasmid pAP3 was recovered, on which a DNA fragment including the promoter region, a part of the prepro sequence and the terminator region of alkali protease was harbored and inserted in between the EcoRI digested site and the HindIII digested site of pUC118. Fig.8 shows the processes of the construction of pAP1, pAP2 and pAP3. Herein, "Papr" represents alkali protease gene promoter; "pre" represents alkali protease pre-sequence; "pro" represents alkali protease pro-sequence; and "ter" represents alkali protease gene terminator.

Digesting plasmids pSMI and pSM2 with XbaI, two DNA fragments carrying the lipase gene were recovered. Linking these two fragments independently to the XbaI site of the plasmid pAP3, plasmids pAP4 and pAP5 were recovered.

Digesting the plasmids pAP4 and pAP5 with EcoRI and HindIII, two DNA fragments carrying the lipase gene were recovered. Linking these two fragments independently to plasmid pHY300PLK (manufactured by TaKaRa Brewery, K.K.), Bacillus subtilis strain SD-800 (the strain with lower protease production potency, produced by the method described in Japanese Patent Laid-open No. Hei 1-141596) was transformed by the protoplast method. Selecting tetracydine-resistant tranformant strains forming a larger clear zone on an agar medium containing 0.5% olive oil emulsion, the plasmid DNA was extracted and purified from these transformants, to recover plasmids pSB1 and pSB2, on which DNA fragments with the promoter region of alkali protease, a part of the prepro sequence thereof, the mature lipase gene or the mature lipase gene along with the gene encoding the protein involved in the production thereof, and the terminator region of alkali protease were harbored and inserted in between the EcoRI digestion site and the HindIII digestion site of pHY300PLK. Figs.9 and 10 depict the processes of the construction of pSB1 and pSB2, respectively (the symbols in the figure are the same as in Figs.1, 6 and 8).

Transforming a protease depletion strain of the Bacillus strain NKS-21 (Accession No.FERM BP-93-1) with the plasmids by the protoplast method, a tetracycline-resistant transformed strain was selected. The protease depletion strain of the Bacillus strain NKS-21 was generated, by helping N-methyl-N'-nitro-N-nitrosoguanidine act on the Bacillus strain NKS-21 and inoculating and culturing the resulting strains on a plate medium containing skimmed milk, and thereafter selecting a strain with no formation of any clear zone.

Example 7

Preparation of lipase

i) Escherichia coli

Culturing independently transformed strains carrying plasmids pSL1 and pSL2 in an L liquid medium (5 ml) containing 50 ppm ampicillin under shaking overnight at 37°C and inoculating 1% of the transformed strains after shaking culture at 37°C for 3 hours onto the same medium (300 ml), isopropyl-beta-thiogalactopyranoside (IPTG) was added therein to a final concentration of 1 mM, to induce the expression of the lac promoter, followed by another 4-hour shaking culture. Centrifuging the culture and collecting the supernatant, a lipase solution was prepared.

ii) Pseudomonas

Culturing transformed strains carrying the plasmids pSP1 and pSP2 under shaking, in a lipase generation medium (300 ml) containing 1% Tween 80 and having been adjusted to pH 9, at 35°C for 14 hours, lipase was generated and secreted in the medium. Centrifuging the culture and collecting the supernatant, a lipase solution was prepared.

After fractionating the solution with ammonium sulfate and removing the ammonium sulfate through dialysis and thereafter treating the resulting solution with a CM cellulose column, the solution was purified as a single band by SDS polyacrylamide electrophoresis.

iii) Bacillus

Culturing transformed strains containing the plasmids pSB1 and pSB2 under shaking in a medium (300 ml) containing 1% casein, 1% meat extract and 1% polypeptone and having been adjusted to pH 7.5 with sodium carbonate at 35°C for 66 hours, lipase was generated and secreted in the medium. Centrifuging the culture and collecting the supernatant, a lipase solution was prepared.

Example 8

Activity of lipase

The activity of the lipase solutions from the cultures of Escherichia coli and Pseudomonas was determined. The assay of the activity was carried out by a method using as the substrate triolein-polyvinyl alcohol (PVA). More specifically, the following method was used.

A mixture solution of 100 mM ε-aminocaproic acid, 100 mM bis-tris[bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane] and 100 mM TAPS [N-tris(hydroxymethyl)methyl-3-aminopropane sulfonic acid], further containing a lipase solution (0.1 ml) and 1 mM calcium chloride, was pH adjusted with sodium hydroxide, and the resulting solution was defined as buffer solution (pH 8.0; 0.4 ml). Heating then a mixture solution of the buffer solution and triolein emulsion (0.5ml) in a stoppered test tube at 37°C for 10 minutes, the reaction was terminated by using 1N hydrochloric acid (0.2 ml) as the reaction termination solution. Triolein was added into 10 ml of an aqueous 2% polyvinyl alcohol (PVA) solution (10 ml) [Poval PVA 117 (manufactured by KURARAY) : Poval PVA 205 (manufactured by KURARAY) = 9 : 1] followed by homogenization, to use herein the resulting mixture as the triolein emulsion. After termination of the reaction, n-hexane (2 ml), isopropyl alcohol (2 ml) and distilled water (1 ml) were added to the reaction mixture under vigorous agitation, and then, the mixture was left to stand. The resulting hexane layer was sampled to assay oleic acid by the TLC-FID method [Minagawa, et. al., Lipids, 18, 732 (1983)]. One unit (1 U) of the activity unit was defined as the enzyme amount generating 1 μmol of oleic acid per one minute.

The activity of each of the lipase solutions (culture supernatant) from the individual transformants is represented as the relative value to the activity of the strain SD 705, which is defined as 100. The activity is shown in Table 1. All the transformants introduced with the lipase gene, expressed the lipase activity under observation; and further, the transformants carrying the plasmids inserted with the DNA fragments including the gene encoding the protein involved in the lipase production, showed higher expression levels of lipase activity than the levels in the transformants carrying only the lipase gene, which indicates that the gene is responsible for the elevation of the lipase production.

Table 1

Lipase Activity	
Plasmid/bacterial strain	Activity
None/JM 101	0
pUC118/JM101	0
pSL1/JM101	20
pSL2/JM101	10
None/SD705	100
pMFY42/SD705	100
pSP1/SD705	520
pSD2/SD705	320
None/LD9	0
pMFY42/LD9	0
pSP1/LD9	110
pSP2/LD9	50

Example 9

Lipase activity

The activity of lipase from the Bacillus culture was assayed. The activity was assayed by the following procedures according to the method using as the substrate p-nitrophenyl palmitate (pNPP)

pNPP was solubilized in isopropyl alcohol to 2 mg/ml. Mixing the pNPP solution and 100 mM Bicine buffer, pH 8.0 at a ratio of 1 : 10, the resulting solution was used as a substrate solution. Adding the lipase solution (0.02 ml) into the substrate solution (0.5 ml), and reacting them together at room temperature for 1 to 10 minutes, 1N HCl (0.2 ml) was added to the reaction mixture for termination of the reaction. Then, the absorbance at 405 nm was measured by a spectrophotometer. One pNPP unit (1 pU) of the enzyme was defined as the enzyme amount increasing the absorbance at 405 nm by one per one minute.

The activities of the lipases from the individual transformants are shown in Table2.

Table 2

Lipase activity	
Plasmid/bacterial strain	Activity
None/SD-800	0
pHY300PLK/SD-800	0
pSB1/SD-800	3.2
pSB2/SD-800	1.1
None/NKS-21	0
pHY300PLK/NKS-21	0
pSB1/NKS-21	5.2
pSB2/NKS-21	2.3

Industrial Applicability

In accordance with the lipase gene of the present invention, the generation and modification of lipase S as a lipid degradation enzyme industrially useful in detergents, paper making, oil manufacturing and the like, are readily carried out. Also, in accordance with the gene encoding the protein involved in the lipase production, in accordance with the present invention, the production of lipase S can be elevated, economically advantageously for providing the industrially useful lipase.

Information of deposited microorganisms

The depository of the microorganisms described in the specification and the claims is described hereinbelow, together with the address and deposition date.

1. Pseudomonas sp. strain SD 705 (Accession No. FERM BP-4772)
Depository: The Life Engineering and Industrial Technology Research Institute, the Agency of industrial Science and Technology, the Ministry of Industry and Trade Address: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan

The strain was deposited as P-13781 on August 4, 1993; under the Budapest Treaty, the strain was transferred and internationally deposited as Pseudomonas sp. strain SD 705 (Accession No. FERM BP-4772) on August 5, 1994.

2. Pseudomonas alcaligenes strain SD 702 (Accession No. FERM BP-4291)
Depository: The Life Engineering and industrial Technology Research Institute, the Agency of Industrial Science and Technology, the Ministry of Industry and Trade Address: 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan

The strain was deposited as P-12944 on May 1, 1992; under the Budapest Treaty, the strain was transferred and internationally deposited as Pseudomonas sp. strain SD 702 (Accession No. FERM BP-4291) on May 12, 1993.

3. Bacillus strain NKS-21 (Accession No. FERM BP-93-1)
Depository: The Microbial industrial Technology Research Institute, the Agency of Industrial Science and Technology, the Ministry of Industry and Trade Address: 1-3, Higashi 1-chome, Yatabe-cho, Tsukuba-gun, Ibaraki-ken, Japan

As the re-deposition of the FERM BP-93, the strain was re-deposited internationally as FERM BP-93-1 on May 21, 1985.

The depository described in the above item 3 has been reorganized currently as "Life Engineering and industrial Technology Research Institute, the Agency of Industrial Science and Technology, the Ministry of Industry and Trade", and the address is modified as 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan.

Sequence Listing

Sequence No.: 1
Sequence length: 288
Sequence type: amino acid
Topology: linear
Sequence species: protein
Origin:
Name of microorganism: Pseudomonas sp.
Name of strain: SD 705
Sequence 1:

```
Phe Gly Ser Ser Asn Tyr Thr Lys Thr Gln Tyr Pro Ile Val Leu Thr
1               5                   10                  15
His Gly Met Leu Gly Phe Asp Ser Leu Leu Gly Val Asp Tyr Trp Tyr
            20                  25                  30
Gly Ile Pro Ser Ala Leu Arg Lys Asp Gly Ala Thr Val Tyr Val Thr
        35                  40                  45
Glu Val Ser Gln Leu Asp Thr Ser Glu Ala Arg Gly Glu Gln Leu Leu
    50                  55                  60
Thr Gln Val Glu Glu Ile Val Ala Ile Ser Gly Lys Pro Lys Val Asn
65                  70                  75                  80
Leu Phe Gly His Ser His Gly Gly Pro Thr Ile Arg Tyr Val Ala Ala
                85                  90                  95
Val Arg Pro Asp Leu Val Ala Ser Val Thr Ser Ile Gly Ala Pro His
            100                 105                 110
Lys Gly Ser Ala Thr Ala Asp Phe Ile Arg Gln Val Pro Glu Gly Ser
        115                 120                 125
Ala Ser Glu Ala Ile Leu Ala Gly Ile Val Asn Gly Leu Gly Ala Leu
    130                 135                 140
Ile Asn Phe Leu Ser Gly Ser Ser Ser Asp Thr Pro Gln Asn Ser Leu
145                 150                 155                 160
Gly Thr Leu Glu Ser Leu Asn Ser Glu Gly Ala Ala Arg Phe Asn Ala
                165                 170                 175
Arg Phe Pro Gln Gly Val Pro Thr Ser Ala Cys Gly Glu Gly Asp Tyr
            180                 185                 190
Val Val Asn Gly Val Arg Tyr Tyr Ser Trp Ser Gly Thr Ser Pro Leu
        195                 200                 205
```

Thr Asn Val Leu Asp Pro Ser Asp Leu Leu Leu Gly Ala Thr Ser Leu
210 215 220
Thr Phe Gly Phe Glu Ala Asn Asp Gly Leu Val Gly Arg Cys Ser Ser
225 230 235 240
Arg Leu Gly Met Val Ile Arg Asp Asn Tyr Arg Met Asn His Leu Asp
245 250 255
Glu Val Asn Gln Thr Phe Gly Leu Thr Ser Ile Phe Glu Thr Ser Pro
260 265 270
Val Ser Val Tyr Arg Gln Gln Ala Asn Arg Leu Lys Asn Ala Gly Leu
275 280 285

Sequence No.: 2
Sequence length: 864
Sequence type: nucleic acid
Chain number: double-stranded
Topology: linear
Sequence species: genomic DNA
Origin:
Name of microorganism: Pseudomonas sp.
Name of strain: SD 705
Sequence characteristics
Symbol representing the characteristics: mat peptide
Existing position: 1..8664
Method for determining the characteristics: E
Sequence

TTC GGC TCC TCG AAC TAC ACC AAG ACC CAG TAC CCG ATC GTC CTG ACC 48
Phe Gly Ser Ser Asn Tyr Thr Lys Thr Gln Tyr Pro Ile Val Leu Thr
1 5 10 15
CAC GGC ATG CTC GGT TTC GAC AGC CTG CTT GGA GTC GAC TAC TGG TAC 96
His Gly Met Leu Gly Phe Asp Ser Leu Leu Gly Val Asp Tyr Trp Tyr
20 25 30
GGC ATT CCC TCA GCC CTG CGT AAA GAC GGC GCC ACC GTC TAC GTC ACC 144
Gly Ile Pro Ser Ala Leu Arg Lys Asp Gly Ala Thr Val Tyr Val Thr
35 40 45
GAA GTC AGC CAG CTC GAC ACC TCC GAA GCC CGA GGT GAG CAA CTG CTG 192
Glu Val Ser Gln Leu Asp Thr Ser Glu Ala Arg Gly Glu Gln Leu Leu

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    50                  55                  60
ACC CAA GTC GAG GAA ATC GTG GCC ATC AGC GGC AAG CCC AAG GTC AAC   240
Thr Gln Val Glu Glu Ile Val Ala Ile Ser Gly Lys Pro Lys Val Asn
65                  70                  75                  80
CTG TTC GGC CAC AGC CAT GGC GGG CCT ACC ATC CGC TAC GTT GCC GCC   288
Leu Phe Gly His Ser His Gly Gly Pro Thr Ile Arg Tyr Val Ala Ala
                85                  90                  95
GTG CGC CCG GAT CTG GTC GCC TCG GTC ACC AGC ATT GGC GCG CCG CAC   336
Val Arg Pro Asp Leu Val Ala Ser Val Thr Ser Ile Gly Ala Pro His
            100                 105                 110
AAG GGT TCG GCC ACC GCC GAC TTC ATC CGC CAG GTG CCG GAA GGA TCG   384
Lys Gly Ser Ala Thr Ala Asp Phe Ile Arg Gln Val Pro Glu Gly Ser
        115                 120                 125
GCC AGC GAA GCG ATT CTG GCC GGG ATC GTC AAT GGT CTG GGT GCG CTG   432
Ala Ser Glu Ala Ile Leu Ala Gly Ile Val Asn Gly Leu Gly Ala Leu
    130                 135                 140
ATC AAC TTC CTT TCC GGC AGC AGT TCG GAC ACC CCA CAG AAC TCG CTG   480
Ile Asn Phe Leu Ser Gly Ser Ser Ser Asp Thr Pro Gln Asn Ser Leu
145                 150                 155                 160
GGC ACG CTG GAG TCA CTG AAC TCC GAA GGC GCC GCA CGG TTT AAC GCC   528
Gly Thr Leu Glu Ser Leu Asn Ser Glu Gly Ala Ala Arg Phe Asn Ala
                165                 170                 175
CGC TTC CCC CAG GGG GTA CCA ACC AGC GCC TGC GGC GAG GGC GAT TAC   576
Arg Phe Pro Gln Gly Val Pro Thr Ser Ala Cys Gly Glu Gly Asp Tyr
            180                 185                 190
GTG GTC AkT GGC GTG CGC TAT TAC TCC TGG AGC GGC ACC ACC CCG CTG   624
Val Val Asn Gly Val Arg Tyr Tyr Ser Trp Ser Gly Thr Ser Pro Leu
        195                 200                 205
ACC AAC GTA CTC GAC CCC TCC GAC CTG CTG CTC GGC GCC ACC TCC CTG   672
Thr Asn Val Leu Asp Pro Ser Asp Leu Leu Leu Gly Ala Thr Ser Leu
    210                 215                 220
ACC TTC GGT TTC GAG GCC AAC GAT GGT CTG GTC GGA CGC TGC AGC TCC   720
Thr Phe Gly Phe Glu Ala Asn Asp Gly Leu Val Gly Arg Cys Ser Ser
225                 230                 235                 240
CGG CTG GGT ATG GTG ATC CGC GAC AAC TAC CGG ATG AAC CAC CTG GAC   768
Arg Leu Gly Met Val Ile Arg Asp Asn Tyr Arg Met Asn His Leu Asp
                245                 250                 255
GAG GTG AAC CAG ACC TTC GGG CTG ACC AGC ATC TTC GAG ACC AGC CCG   816
```

Glu Val Asn Gln Thr Phe Gly Leu Thr Ser Ile Phe Glu Thr Ser Pro
260 265 270

GTA TCG GTC TAT CGC CAG CAA GCC AAT CGC CTG AAG AAC GCC GGG CTC 864
Val Ser Val Tyr Arg Gln Gln Ala Asn Arg Leu Lys Asn Ala Gly Leu
275 280 285

Sequence No.: 3

Sequence length: 335

Sequence type: amino acid

Topology: linear

Sequence species: protein

Origin:

Name of microorganism: Pseudomonas sp.

Name of strain: SD 705

Sequence

Met Lys Pro Leu Ile Tyr Leu Pro Leu Leu Leu Gly Leu Gly Leu Leu
1 5 10 15

Gly Trp His Leu Ser Thr Pro Ala Pro Ser Pro Ser Ser Ala Ser Pro
20 25 30

Ala Pro Pro Gln Val Ser Ser Glu Lys Pro Ala Thr Ala His Met Asp
35 40 45

Leu Thr Arg Pro Val Ala Arg Ser Thr Asp Gln His Leu Pro Ala Ser
50 55 60

Leu Arg Asp Thr Asp Val Asp Gly Gln Leu Glu Val Asp Ala Gln Gly
65 70 75 80

Asn Leu Val Ile Ser Asp Gln Leu Arg His Leu Phe Asp Tyr Phe Phe
85 90 95

Ser Thr Val Gly Glu Gln Ser Phe Glu Gln Ala Ser Thr Gly Ile Arg
100 105 110

Asp Tyr Leu Ala Ser Gln Leu Arg Glu Pro Ala Leu Gly Gln Ala Leu
115 120 125

Asp Leu Leu Asp Arg Tyr Ile Asn Tyr Lys Thr Glu Leu Val Glu Leu
130 135 140

Glu Arg Arg Phe Pro Met Val Thr Glu Leu Asp Gly Leu Arg Ala Arg
145 150 155 160

Glu Asp Ala Val Gln Arg Leu Arg Ala Ser Leu Phe Asn Ala Gln Glu

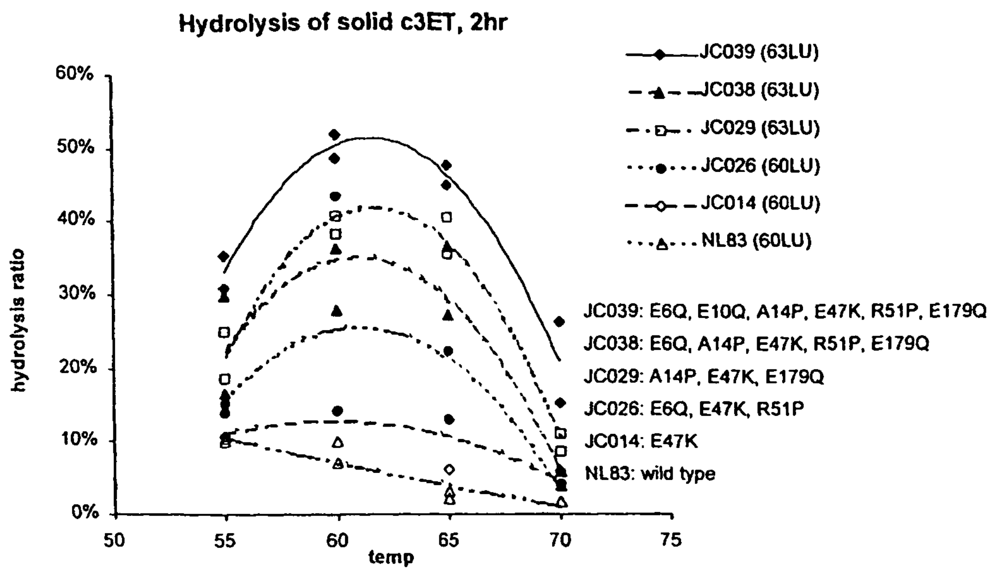

Fig. 3

Hydrolysis of solid c3ET, 2 hr

165 170 175

His Ala Ala Phe Phe Ala Ser Glu Glu Val Tyr Asn Gln Phe Thr Leu

180 185 190

Glu Arg Leu Ala Ile Leu His Asp Pro Ser Leu Asp Pro Gln Asp Lys

195 200 205

Ala Glu Arg Ile Glu Arg Leu Arg Glu Gly Leu Pro Asp Glu Leu Gln

210 215 220

Gln Leu Leu Val Pro Gln Leu His Leu Thr Leu Arg Gln Gln Thr Gln

225 230 235 240

Gln Leu Leu Glu Gln Gly Ala Glu Pro Glu Gln Leu Arg Gln Leu Arg

245 250 255

Leu Asn Leu Val Gly Pro Gln Ala Thr Glu Arg Leu Glu Ala Leu Asp

260 265 270

Arg Gln Arg Ser Glu Trp Asp Gln Arg Leu Ser Gly Phe Asn Arg Glu

275 280 285

Arg Gln Ala Ile Ile Ser Gln Pro Gly Leu Ala Asp Ser Asp Lys Gln

290 295 300

Ala Ala Ile Glu Ala Leu Leu His Glu Gln Phe Ser Glu His Glu Arg

305 310 315 320

Leu Arg Val Ser Ser Leu Leu Gly Leu Asp Ser Arg Ala Glu Arg

325 330 335

Sequence No.: 4

Sequence length: 1005

Sequence type: nucleic acid

Chain number: double-stranded

Topology: linear

Sequence species: genomic DNA

Origin:

Name of microorganism: Pseudomonas sp.

Name of strain: SD 705

Sequence characteristics

Symbol representing the characteristics: mat peptide

Existing position: 1..1005

Method for determining the characteristics: E

Sequence

ATG AAG CCG CTG ATT TAT CTG CCT TTG CTT CTT GGC CTG GGG CTG CTC 48
Met Lys Pro Leu Ile Tyr Leu Pro Leu Leu Leu Gly Leu Gly Leu Leu
1 5 10 15
GGC TGG CAC CTG AGC ACG CCG GCA CCC AGC CCA TCC AGC GCC TCA CCA 96
Gly Trp His Leu Ser Thr Pro Ala Pro Ser Pro Ser Ser Ala Ser Pro
20 25 30
GCG CCG CCA CAA GTC AGC AGT GAA AAA CCT GCC ACG GCT CAC ATG GAC 144
Ala Pro Pro Gln Val Ser Ser Glu Lys Pro Ala Thr Ala His Met Asp
35 40 45
CTG ACC CGC CCG GTG GCC CGC AGC ACC GAC CAG CAT CTG CCC GCC TCG 192
Leu Thr Arg Pro Val Ala Arg Ser Thr Asp Gln His Leu Pro Ala Ser
50 55 60
CTG CGC GAT ACC GAC GTC GAT GGC CAG CTG GAG GTC GAC GCC CAG GGC 240
Leu Arg Asp Thr Asp Val Asp Gly Gln Leu Glu Val Asp Ala Gln Gly
65 70 75 80
AAT CTG GTG ATT TCC GAC CAG CTG CGC CAC CTG TTC GAC TAT TTC TTC 288
Asn Leu Val Ile Ser Asp Gln Leu Arg His Leu Phe Asp Tyr Phe Phe
85 90 95
AGC ACC GTC GGC GAA CAG TCG TTC GAG CAG GCC AGC ACC GGT ATC CGC 336
Ser Thr Val Gly Glu Gln Ser Phe Glu Gln Ala Ser Thr Gly Ile Arg
100 105 110
GAC TAT CTG GCC AGC CAG CTG CGT GAA CCG GCT CTG GGT CAG GCC CTG 384
Asp Tyr Leu Ala Ser Gln Leu Arg Glu Pro Ala Leu Gly Gln Ala Leu
115 120 125
GAT CTG CTG GAT CGC TAT ATC AAC TAC AAG ACC GAG CTG GTG GAA CTG 432
Asp Leu Leu Asp Arg Tyr Ile Asn Tyr Lys Thr Glu Leu Val Glu Leu
130 135 140
GAG CGA CGC TTC CCG ATG GTG ACC GAG CTG GAC GGC CTG CGT GCC CGT 480
Glu Arg Arg Phe Pro Met Val Thr Glu Leu Asp Gly Leu Arg Ala Arg
145 150 155 160
GAA GAT GCC GTA CAG CGC CTG CGC GCC AGC CTG TTC AAC GCG CAG GAG 528
Glu Asp Ala Val Gln Arg Leu Arg Ala Ser Leu Phe Asn Ala Gln Glu
165 170 175
CAC GCC GCC TTC TTC GCC AGC GAA GAG GTC TAT AAC CAG TTC ACT CTT 576
His Ala Ala Phe Phe Ala Ser Glu Glu Val Tyr Asn Gln Phe Thr Leu
180 185 190
GAG CGT CTG GCG ATA CTG CAC GAC CCG TCG CTG GAT CCG CAG GAC AAG 624
Glu Arg Leu Ala Ile Leu His Asp Pro Ser Leu Asp Pro Gln Asp Lys

195 200 205
GCC GAG CGG ATC GAA CGT CTG CGC GAA GGG CTA CCC GAC GAG TTG CAA 672
Ala Glu Arg Ile Glu Arg Leu Arg Glu Gly Leu Pro Asp Glu Leu Gln
210 215 220
CAA TTG CTG GTA CCG CAA TTA CAC CTG ACC CTG CGC CAG CAG ACC CAG 720
Gln Leu Leu Val Pro Gln Leu His Leu Thr Leu Arg Gln Gln Thr Gln
225 230 235 240
CAG TTG CTG GAG CAA GGC GCC GAG CCG GAA CAG CTA CGC CAA TTG CGC 768
Gln Leu Leu Glu Gln Gly Ala Glu Pro Glu Gln Leu Arg Gln Leu Arg
245 250 255
CTG AAC CTG GTC GGC CCC CAG GCA ACC GAA CGC CTG GAG GCA CTG GAC 816
Leu Asn Leu Val Gly Pro Gln Ala Thr Glu Arg Leu Glu Ala Leu Asp
260 265 270
CGC CAG CGC AGC GAA TGG GAT CAG CGC CTG AGC GGC TTC AAT CGC GAA 864
Arg Gln Arg Ser Glu Trp Asp Gln Arg Leu Ser Gly Phe Asn Arg Glu
275 280 285
CGG CAG GCG ATC ATC AGC CAG CCG GGG CTG GCC GAC AGT GAC AAG CAG 912
Arg Gln Ala Ile Ile Ser Gln Pro Gly Leu Ala Asp Ser Asp Lys Gln
290 295 300
GCC GCG ATT GAG GCC CTG CTG CAC GAG CAG TTC AGT GAG CAT GAG CGG 960
Ala Ala Ile Glu Ala Leu Leu His Glu Gln Phe Ser Glu His Glu Arg
305 310 315 320
CTG AGG GTC AGC AGT CTG CTG GGA CTC GAT AGC CGC GCC GAA CGC 1005
Leu Arg Val Ser Ser Leu Leu Gly Leu Asp Ser Arg Ala Glu Arg
325 330 335

Sequence No.: 5

Sequence length: 26

Sequence type: amino acid

Topology: linear

Sequence species: peptide

Fragment type: N-terminal fragment

Origin:

Name of microorganism: Pseudomonas sp.

Name of strain: SD 705

Sequence

Phe Gly Ser Ser Asn Tyr Thr Lys Thr Gln Tyr Pro Ile Val Leu Thr

1 5 10 15
His Gly Met Leu Gly Phe Asp Ser Leu Leu
20 25

Sequence No.: 6

Sequence length: 20
Sequence type: nucleic acid
Chain number: single-stranded
Topology: linear
Sequence species: other nucleic acid, synthetic DNA
Sequence

AACTACACNA AGACNCAGTA 20

Sequence No.: 7

Sequence length: 30
Sequence type: nucleic acid
Chain number: single-stranded
Topology: linear
Sequence species: other nucleic acid, synthetic DNA
Sequence

GGGGAATTCA GGACTCGCAT TATGCGCAAC 30

Sequence No.: 8

Sequence length: 30
Sequence type: nucleic acid
Chain number: single-stranded
Topology: linear
Sequence species: other nucleic acid, synthetic DNA
Sequence

TTTGAATTCA GAGCCCGGCG TTCTTCAGGC 30

Sequence No.: 9

Sequence length: 30

Sequence type: nucleic acid
Chain number: single-stranded
Topology: linear
Sequence species: other nucleic acid, synthetic DNA
Sequence

TTTGAGCTCA GAGCCCGGCG TTCTTCAGGC 30

Sequence No.: 10
Sequence length: 30
Sequence type: nucleic acid
Chain number: single-stranded
Topology: linear
Sequence species: other nucleic acid, synthetic DNA
Sequence

AAATCTAGAT TCGGCTCCTC GAACTACACC 30

Sequence No.: 11
Sequence length: 30
Sequence type: nucleic acid
Chain number: single-stranded
Topology: linear
Sequence species: other nucleic acid, synthetic DNA
Sequence

CCCTCTAGAC TAGCGTTCGG CGCGGCTATC 30

Sequence No.: 12
Sequence length: 30
Sequence type: nucleic acid
Chain number: single-stranded
Topology: linear
Sequence species: other nucleic acid, synthetic DNA
Sequence

CCCTCTAGAT CAGAGCCCGG CGTTCTTCAG 30

Sequence No.: 13

Sequence length: 30

Sequence type: nucleic acid

Chain number: single-stranded

Topology: linear

Sequence species: other nucleic acid, synthetic DNA

Sequence

CCCGAATTCA TACGAATTAA AGTTGAAAGC 30

Sequence No.: 14

Sequence length: 30

Sequence type: nucleic acid

Chain number: single-stranded

Topology: linear

Sequence species: other nucleic acid, synthetic DNA

Sequence

AAATCTAGAG TTGAAACCAA TTAAGTACTC 30

Sequence No.: 15

Sequence length: 30

Sequence type: nucleic acid

Chain number: single-stranded

Topology: linear

Sequence species: other nucleic acid, synthetic DNA

Sequence

GGGTCTAGAT CCCTAAGGAT GTACTGGATG 30

Sequence No.: 16

Sequence length: 30
Sequence type: nucleic acid
Chain number: single-stranded
Topology: linear
Sequence species: other nucleic acid, synthetic DNA
Sequence

TTTAAGCTTA GAAACTCAAC TGTCACAGTG 30

Sequence Listing

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45-4444-8888
(H) TELEFAX: +45-4449-3256

(ii) TITLE OF INVENTION: Novel Lipase Gene and Method for Producing lipase using the same

(iii) NUMBER OF SEQUENCES: 16

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 288 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Pseudomonas sp.
(B) STRAIN: SD 705

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Phe Gly Ser Ser Asn Tyr Thr Lys Thr Gln Tyr Pro Ile Val Leu Thr
1               5                   10                  15
His Gly Met Leu Gly Phe Asp Ser Leu Leu Gly Val Asp Tyr Trp Tyr
            20                  25                  30
Gly Ile Pro Ser Ala Leu Arg Lys Asp Gly Ala Thr Val Tyr Val Thr
        35                  40                  45
Glu Val Ser Gln Leu Asp Thr Ser Glu Ala Arg Gly Glu Gln Leu Leu
    50                  55                  60
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Thr Gln Val Glu Glu Ile Val Ala Ile Ser Gly Lys Pro Lys Val Asn
65   70   75   80

Leu Phe Gly His Ser His Gly Gly Pro Thr Ile Arg Tyr Val Ala Ala
                85   90   95

Val Arg Pro Asp Leu Val Ala Ser Val Thr Ser Ile Gly Ala Pro His
            100   105   110

Lys Gly Ser Ala Thr Ala Asp Phe Ile Arg Gln Val Pro Glu Gly Ser
        115   120   125

Ala Ser Glu Ala Ile Leu Ala Gly Ile Val Asn Gly Leu Gly Ala Leu
    130   135   140

Ile Asn Phe Leu Ser Gly Ser Ser Ser Asp Thr Pro Gln Asn Ser Leu
145   150   155   160

Gly Thr Leu Glu Ser Leu Asn Ser Glu Gly Ala Ala Arg Phe Asn Ala
                165   170   175

Arg Phe Pro Gln Gly Val Pro Thr Ser Ala Cys Gly Glu Gly Asp Tyr
            180   185   190

Val Val Asn Gly Val Arg Tyr Tyr Ser Trp Ser Gly Thr Ser Pro Leu
        195   200   205

Thr Asn Val Leu Asp Pro Ser Asp Leu Leu Leu Gly Ala Thr Ser Leu
    210   215   220

Thr Phe Gly Phe Glu Ala Asn Asp Gly Leu Val Gly Arg Cys Ser Ser
225   230   235   240

Arg Leu Gly Met Val Ile Arg Asp Asn Tyr Arg Met Asn His Leu Asp
                245   250   255

Glu Val Asn Gln Thr Phe Gly Leu Thr Ser Ile Phe Glu Thr Ser Pro
            260   265   270

Val Ser Val Tyr Arg Gln Gln Ala Asn Arg Leu Lys Asn Ala Gly Leu
        275   280   285

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 864 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

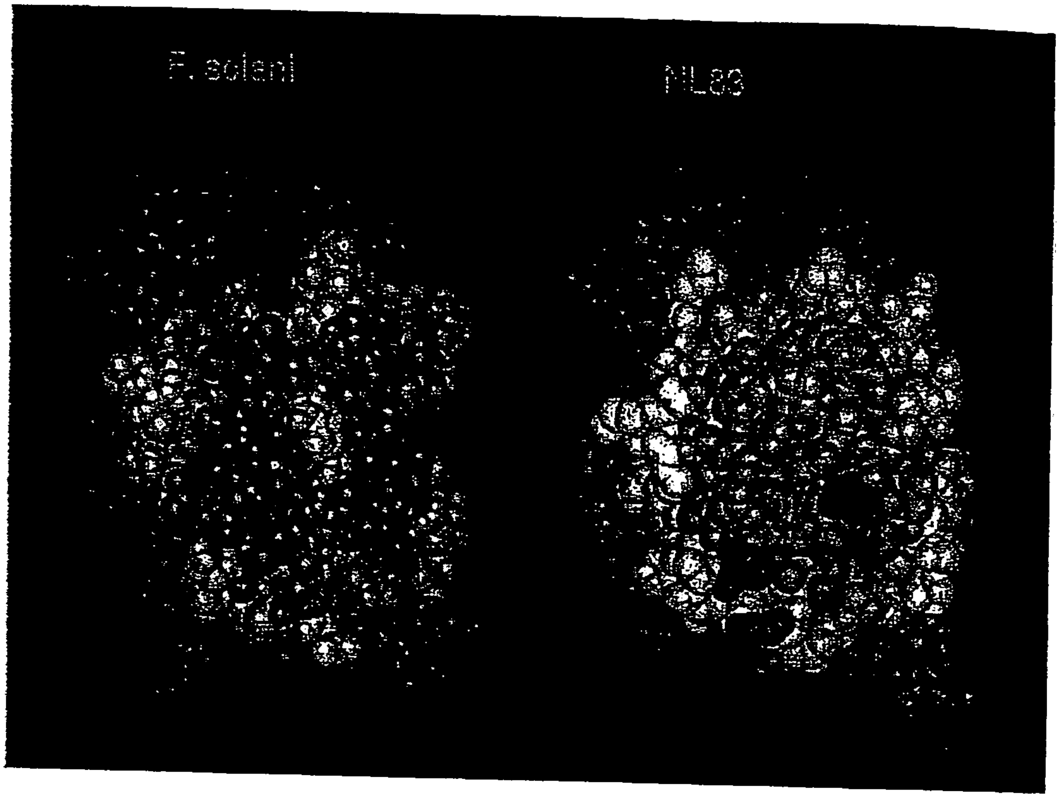

Fig. 2

3D structure of cutinases from *F. solani pisi* (left) and *H. insolens* (right)

ATOM	210	OD1	ASN	A	33	16.112	-10.395	17.089	1.00	19.05
ATOM	211	ND2	ASN	A	33	18.074	-10.460	16.112	1.00	13.29
ATOM	212	N	MET	A	34	19.633	-8.378	14.282	1.00	14.22
ATOM	213	CA	MET	A	34	20.282	-7.171	14.751	1.00	12.97
ATOM	214	C	MET	A	34	21.142	-6.663	13.611	1.00	19.02
ATOM	215	O	MET	A	34	21.654	-5.512	13.713	1.00	26.04
ATOM	216	CB	MET	A	34	21.202	-7.329	15.859	1.00	13.39
ATOM	217	CG	MET	A	34	20.579	-7.713	17.163	1.00	9.02
ATOM	218	SD	MET	A	34	20.175	-6.316	18.069	1.00	9.13
ATOM	219	CE	MET	A	34	21.481	-5.121	18.095	1.00	4.11
ATOM	220	N	GLY	A	35	21.259	-7.446	12.550	1.00	19.99
ATOM	221	CA	GLY	A	35	22.071	-7.135	11.418	1.00	14.30
ATOM	222	C	GLY	A	35	23.511	-7.340	11.764	1.00	17.58
ATOM	223	O	GLY	A	35	23.965	-7.724	12.842	1.00	12.78
ATOM	224	N	ILE	A	36	24.450	-6.839	10.950	1.00	20.63
ATOM	225	CA	ILE	A	36	25.833	-7.029	11.277	1.00	17.71
ATOM	226	C	ILE	A	36	26.609	-5.714	11.280	1.00	16.15
ATOM	227	O	ILE	A	36	27.865	-5.618	11.662	1.00	20.30
ATOM	228	CB	ILE	A	36	26.412	-8.070	10.327	1.00	30.19
ATOM	229	CG1	ILE	A	36	26.088	-7.448	8.959	1.00	31.16
ATOM	230	CG2	ILE	A	36	25.944	-9.490	10.543	1.00	15.68
ATOM	231	CD1	ILE	A	36	26.922	-8.149	7.958	1.00	34.10
ATOM	232	N	THR	A	37	25.905	-4.589	11.040	1.00	13.00
ATOM	233	CA	THR	A	37	26.825	-3.396	11.141	1.00	9.67
ATOM	234	C	THR	A	37	26.587	-2.513	12.350	1.00	15.44
ATOM	235	O	THR	A	37	27.040	-3.055	13.410	1.00	20.20
ATOM	236	CB	THR	A	37	26.592	-2.679	9.818	1.00	14.13
ATOM	237	OG1	THR	A	37	25.241	-2.212	9.503	1.00	22.62
ATOM	238	CG2	THR	A	37	26.949	-3.739	8.800	1.00	2.29
ATOM	239	N	VAL	A	38	25.733	-1.493	12.249	1.00	11.92
ATOM	240	CA	VAL	A	38	25.237	-0.800	13.411	1.00	15.22
ATOM	241	C	VAL	A	38	24.588	-1.455	14.612	1.00	14.68
ATOM	242	O	VAL	A	38	24.906	-1.185	15.733	1.00	15.89
ATOM	243	CB	VAL	A	38	24.124	0.180	12.855	1.00	14.13
ATOM	244	CG1	VAL	A	38	23.663	0.897	14.167	1.00	13.55
ATOM	245	CG2	VAL	A	38	24.570	1.025	11.670	1.00	6.75
ATOM	246	N	GLY	A	39	23.745	-2.410	14.677	1.00	14.24
ATOM	247	CA	GLY	A	39	23.135	-3.151	15.746	1.00	11.03
ATOM	248	C	GLY	A	39	24.096	-3.586	16.791	1.00	13.34
ATOM	249	O	GLY	A	39	24.131	-3.181	17.934	1.00	15.13
ATOM	250	N	PRO	A	40	25.067	-4.340	16.352	1.00	14.70
ATOM	251	CA	PRO	A	40	26.094	-5.025	17.171	1.00	13.44
ATOM	252	C	PRO	A	40	27.010	-3.909	17.589	1.00	11.81
ATOM	253	O	PRO	A	40	27.346	-3.871	18.764	1.00	12.79
ATOM	254	CB	PRO	A	40	26.723	-6.111	16.279	1.00	8.43
ATOM	255	CG	PRO	A	40	25.873	-6.243	14.950	1.00	4.84
ATOM	256	CD	PRO	A	40	25.198	-4.902	14.995	1.00	12.36
ATOM	257	N	ALA	A	41	27.226	-2.979	16.695	1.00	7.41
ATOM	258	CA	ALA	A	41	28.066	-1.962	17.278	1.00	11.03
ATOM	259	C	ALA	A	41	27.378	-1.206	18.439	1.00	14.87
ATOM	260	O	ALA	A	41	28.028	-0.503	19.274	1.00	14.26
ATOM	261	CB	ALA	A	41	28.579	-0.905	16.313	1.00	7.17
ATOM	262	N	LEU	A	42	26.135	-0.811	18.237	1.00	11.87

recombinant vector carrying these genes; a transformant through transformation with such recombinant vector; and a method for producing the lipase S from such transformant.

Brief Description of the Drawings

Fig.1 is a restriction map of plasmid pS1.
Fig.2 is a restriction map of plasmid pS1S.
Fig.3 is a restriction map of plasmid pS1E.
Fig.4 is a restriction map of plasmid pSL1.
Fig.5 is a restriction map of plasmid pSL2.
Fig.6 is a restriction map of plasmid pSP1.
Fig.7 is a restriction map of plasmid pSP2.
Fig.8 depicts the constructions of plasmids pAP1, pAP2 and pAP3.
Fig.9 depicts the construction of plasmid pSB1.
Fig.10 depicts the construction of plasmid pSB2.

Detailed Description of the Invention

From Pseudomonas sp. strain SD 705 (Accession No. FERM BP-4772) firstly deposited by the present inventors at the Life Engineering and Industrial Technology Research Institute, the Agency of Industrial Science and Technology, at 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan and then transferred under the Budapest Treaty to international deposition, the present inventors have isolated a DNA fragment carrying the gene encoding the lipase S and the gene encoding the protein involved in the production. The strain SD 705 of the genus Pseudomonas belongs to lipase S-producing bacteria useful for detergent and the like. Introducing the DNA fragment into a host cell and culturing the resultant transformant in a culture medium, it has been confirmed that lipase S was generated in the culture. Thus, the present invention has been achieved.

More specifically, the present invention is to provide what will be described below.

1. A gene encoding the amino acid sequence of Sequence No. 1.
2. A gene carrying the lipase-encoding nucleotide sequence of Sequence No.2.
3. A gene encoding the amino acid sequence of Sequence No.3.
4. A gene carrying the nucleotide sequence of Sequence No. 4, which encodes the protein involved in the lipase production.
5. A gene described in any one of 1 to 4, which is derived from bacteria of the genus Pseudomonas.
6. A gene described in any one of 1 to 4, which is derived from Pseudomonas sp. strain SD 705 (Accession No. FERM BP-4772).
7. A DNA containing the whole or a part of the nucleotide sequence of the gene described in any one of 1 to 6.
8. A DNA hybridizable with the whole or a part of the nucleotide sequence of the gene described in 3 or 4.
9. A recombinant DNA inserted into a vector replicable in a host microbial cell, so that at least one of the genes described in 1 to 4 might be expressed in the host microbial cell.
10. A recombinant chromosomal DNA generated by incorporating at least one of the genes described in 1 to 4 into a microbial chromosome via homologous recombination.
11. A transformed host microorganism, having been transformed with the recombinant DNA described in 9.
12. A transformed microorganism carrying the recombinant chromosomal DNA described in 10.
13. A transformed microorganism described in 11, wherein the microorganism is a bacterium of the genus Escherichia, Pseudomonas or Bacillus.
14. A transformed microorganism described in 12, wherein the microorganism is a bacterium of the genus Pseudomonas or Bacillus.
15. A transformed microorganism described in 11 or 12, wherein the microorganism is Pseudomonas alcaligenes, Pseudomonas pseudoalcaligenes, Pseudomonas mendocina, or Bacillus subutilis.
16. A transformed microorganism described in 11 or 12, wherein the microorganism is Pseudomonas sp. strain SD 705 (Accession No.FERM BP-4772), Pseudomonas mendocina strain SD 702 (Accession No. FERMBP-4291), Bacillus strain NKS-21 (Accession NO.FERM BP-93-1) or a variant thereof.
17. A method for producing the lipase, comprising culturing at least one of the transformed host cells described in 11 to 16 to produce a culture containing the lipase and isolating the lipase.

The present invention will now be described hereinbelow in detail.

Isolation of gene

In accordance with the present invention, the lipase-encoding gene can be isolated from chromosomal DNAs by known methods such as colony hybridization and the formation of a clear zone on a plate medium. More specifically, firstly, a chromosome library is prepared. If the whole or a part of the amino acid sequence of the lipase is known, an oligonucleotide probe corresponding to the whole or a part of the sequence is then prepared and using the probe, the gene encoding the lipase can be isolated through colony hybridization.

When the amino acid sequence of the lipase absolutely is not known, an oligonucleotide corresponding to a sequence around the active center residue may be used as the probe, which sequence has generally been known to be highly preserved in lipases from microbial organisms.

Otherwise, using as primers oligonucleotides independently corresponding to two different preserved sequences and as a template a chromosome with an objective lipase gene, the sequence between the two primers is enzymatically synthesized via DNA polymerase, to prepare a double-stranded DNA, of which both the strands may be used as the probes.

Otherwise, a chromosome library is prepared in a bacterium with no lipase production potency, and is then cultured in an agar plate containing a sparingly soluble substrate of lipase. A bacterium containing a chromosomal DNA fragment carrying the lipase gene decomposes the substrate around the colony, so that the screening of the bacterium can be conducted on the basis of the formation of a clear zone. By this method, the gene encoding the lipase may be isolated, but any of these methods may be used satisfactorily.

By colony hybridization using as the probe the whole of the lipase-encoding gene or a part of the 3' region thereof, the gene encoding the protein involved in the lipase production may be isolated as a DNA fragment downstream the lipase gene, from the chromosomal DNA.

Host

Any host capable of expressing the isolated gene may be used as the host to introduce the gene, including for example bacteria of genera Pseudomonas, Escherichia, and Bacillus.

Bacteria of genus Pseudomonas are preferably Pseudomonas sp. strain SD 705 (Accession No. FERM BP-4772) or variants thereof, Pseudomonas alcaligenes , Pseudomonas pseudoalcaligenes and Pseudomonas mendocina. More preferably, the bacteria are Pseudomonas sp. strain SD 705 or variants thereof, Pseudomonas mendocina strain SD 702 (Accession No. FERM BP-4291) or variants thereof.

As the bacteria of genus Escherichia, preference is given to Escherichia coli.

Bacteria of genus Bacillus are preferably Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus firmus, Bacillus lentus, and Bacillus alcalophilus. More preferably, the bacteria belong to Bacillus sp. strain NKS-21 (Accession No. FERM BP-93-1) or variants thereof.

Transformation

The recovered lipase gene and the gene encoding the protein responsible for the production of the lipase are introduced into a host bacterium. These two genes are linked concurrently to one vector so that the same sequence as in the chromosome can be regenerated or the genes are independently linked to two vectors capable of coexisting in a host cell.

In a host except the bacteria of genus Pseudomonas, if used, the genes are inserted and linked between a promoter and signal sequence and a terminator, both functioning in the host, so that the genes can be expressed in the host. Using a recombinant vector with the genes linked therein, a host bacterium is transformed. When the two genes are linked to different vectors, only the vector carrying the lipase gene singly may be used for transformation or the two recombinant vectors are simultaneously used for transformation. In Escherichia coli if used as a host, for example, plasmids of pUC or pACYC can be used. In bacteria of genus Bacillus, if used as hosts, plasmids of pUB110 and pE194 can be used.

In bacteria of genus Pseudomonas if used as hosts, plasmids of RSF1010, etc. can be used. The genes can thereby be stably harbored outside the chromosomes of the host bacteria. The genes can also be introduced therein by a method for incorporating a DNA of non-replicable form in a host bacterium into the chromosome.

Generation of lipase

In bacteria of genus Pseudomonas if used as hosts, the lipase is secreted into the culture broth. The separation and purification of the lipase from the culture broth can be carried out by adding ammonium sulfate to the culture broth, fractionating crude lipase, removing the ammonium sulfate through dialysis, and isolating the lipase through a CM cellulose column, to purify the lipase as a single band by SDS polyacrylamide gel electrophoresis. However, the lipase

Description

Technical Field

The present invention relates to a novel gene encoding a lipase as a lipid degradation enzyme industrially useful for detergents, food processing, paper making, oil manufacturing or the like and the nucleotide sequence thereof; a gene encoding the protein involved in the production of the lipase and the nucleotide sequence thereof; a recombinant vector carrying these genes, a transformant carrying the recombinant vector; and a method for producing the lipase using the transformant.

Background Art

As lipase-producing microorganisms, there have been known genus Pseudomonas, genus Alcaligenes, genus Mucor, genus Candida, genus Humicola, genus Rhizomucor and the like. Genes have been isolated from some of them, and a great number of lipase genes have been isolated from microorganisms of the genus Pseudomonas, in particular. Currently known such lipase genes include those from Pseudomonas fragi (Japanese Patent Laid-open Nos. Sho 62-228279 and Hei 2-190188), Pseudomonas cepacia (Japanese Patent Laid-open Nos. Hei 3-47079 and Hei 3-87187), Pseudomonas putida (EP 268 452), Pseudomonas pseudoalcaligenes (Japanese Patent Laid-open No. Hei 3-500845), Pseudomonas aeruginosa (EP 334 462), Pseudomonas glumae (Appl. Envir. Microbiol. (1992), 3738 - 3791), and Pseudomonas fluorescens (Appl. Envir. Microbiol. (1992) 58, 1776 - 1779).

It has been known that a protein encoded by a gene region downstream the lipase structural gene is involved in the lipase production in some bacteria of the genus Pseudomonas. For lipase production in Pseudomonas cepacia, the gene in the downstream region is essential, irrespective of the species of a host bacterium (EP 331 376). It has been known also that irrespective of the species of a host bacterium, the protein with an effect of stabilizing lipase is encoded by the region in the lipase produced from Pseudomonas glumae (EP 464 922).

Alternatively, a homologous host-vector system of Pseudomonas pseudoalcaligenes exerts an effect of elevating lipase production, but the gene in the downstream region is not essential for lipase production in a heterologus host-vector system thereof (EP 334 462). Furthermore, the gene in the downstream region is not present in Pseudomonas fragi.

It has been known conventionally that the washing effect of a detergent can be elevated when the detergent is blended with a lipase to degrade and remove the lipid attached to articles to be washed. The use is described in H. Andree, et. al., "Lipase as Detergent Components", Journal of Applied Biochemistry, 2, 218 - 229 (1980) and the like.

Preferably, a lipase to be blended with detergents can satisfactorily exert its lipase activity in a detergent solution. Under routine washing conditions, the pH of washing solutions reside in an alkaline region, so a lipase functioning at an alkaline pH is demanded. Additionally, it has been known that lipid stain is relatively readily removed generally under high temperature and high alkaline conditions but cannot sufficiently be removed through washing at low temperatures (at 60 or less). Not only in Japan where washing has conventionally been carried out at low temperatures, but also in European countries and USA, the washing temperature is likely lowered. Thus, preferably, a lipase to be blended in detergents should satisfactorily function even at low temperatures. Additionally, it is preferable that the lipase to be blended into detergents should sufficiently exhibit its functions during washing even in the presence of detergent components such as surfactant, and protease or bleach contained in many of detergents. Furthermore, preferably, the lipase to be blended into detergents should be stable in the concurrent presence of components contained in the detergents even when the lipase is stored in a blended state in the detergents.

As lipase-producing microorganisms, there have been known the genus Pseudomonas, the genus Alcaligenes, the genus Achromobacter, the genus Mucor, the genus Candida, the genus Humicola, the genus Rhizomucor and the like. Because most of the lipases from these bacterial strains have an optimum pH in a neutral to mild alkaline region, the lipases cannot work sufficiently in alkaline detergent solutions or are poorly stable therein. Still furthermore, the individual lipases from the genus Achromobacter, the genus Mucor, the genus Candida, and the genus Humicola are strongly inhibited of their activities in the presence of anionic surfactants.

Lipase-producing bacteria of the genus Pseudomonas include Pseudomonas fragi, Pseudomonas cepacia, Pseudomonas pseudoalcaligenes, Pseudomonas aeruginosa, and Pseudomonas fluorescens, but known enzymes having been isolated from these bacterial strains cannot satisfy the properties described above.

Disclosure of the Invention

The present invention relates to a method for efficiently producing a lipase with excellent properties for use in industrial fields such as detergent industry, particularly lipase S from a strain SD 705 (FERM BP-4772).

More specifically, the present invention relates to a gene encoding the lipase S and the nucleotide sequence thereof; a gene encoding a protein involved in the production of the lipase S and the nucleotide sequence thereof; a

Example 6

Construction of recombinant vector and preparation of transformant

i) Escherichia coli

An oligonucleotide of Sequence No.7 was chemically synthesized, which worked to add the EcoRI site to the 5' terminus of a sequence corresponding to a segment containing the ribosome linking site (SD sequence) and the initiation codon of the lipase gene. Mixing two oligonucleotides, namely the oligonucleotide and commercially available M13 primer RV (manufactured by TaKaRa Brewery K.K.), with the plasmid pS1E for polymerase chain reaction (POLYMERASE CHAIN REACTION (PCR)), a DNA fragment carrying the lipase gene including the SD sequence and the gene encoding the protein involved in the production thereof was generated. After completely digesting the fragment with EcoRI and fractionating the digested products by agarose gel electrophoresis, the products were extracted and purified from the agarose gel. After completely digesting the plasmid pUC118 with EcoRI and mixing the resulting digested products with the DNA fragment thus purified for linking them together with T4 DNA ligase for transforming the Escherichia coli strain JM 101, ampicillin-resistant transformants were isolated. From these transformants, the plasmid DNA was extracted, purified and analyzed, so as to confirm that the transformants thus produced carried a plasmid on which the DNA fragment carrying the lipase gene and the gene encoding the protein involved in the production thereof was harbored and inserted into the EcoRI restriction site of pUC118, to express the lipase gene and the gene encoding the protein involved in the production thereof downstream the lac promoter. The plasmid is defined as pSL1. Fig.4 shows the restriction map of pSL1 (the symbols in the figure are the same as in Fig.1).

An oligonucleotide to add the EcoRI site to the 3' terminus of the lipase gene, as shown as Sequence No.8, was chemically synthesized. Mixing the oligonucleotide and the oligonucleotide preliminary synthesized, as shown as Sequence No. 7, with the plasmid pS1E for polymerase chain reaction (PCR), a DNA fragment containing only the lipase gene including the SD sequence was recovered. The fragment was completely digested with EcoRI, and was then linked with the plasmid pUC118 in the same manner as described above for transformation, to recover a transformant carrying a plasmid pSL2 on which the DNA fragment containing only the lipase gene was harbored and inserted into the EcoRI restriction site of pUC118 to express the lipase gene downstream the lac promoter. Fig.5 shows the restriction map of the pSL2 (the symbols in the figure are the same as in Fig.1).

ii) Pseudomonas

After completely digesting the plasmid pS1S with HindIII and SacI, a DNA fragment containing the lipase gene and the gene encoding the protein involved in the production thereof was fractionated by agarose gel electrophoresis followed by extraction and purification from the agarose gel. After completely digesting the plasmid pMFY42 with the HindIII and SacI, the resulting products were mixed with the DNA fragment thus purified, followed by linking with T4 DNA ligase to transform Escherichia coli strain JM 101 to select kanamycin-resistant colonies. From these transformants, the plasmid DNA was extracted, purified and analyzed, to recover a plasmid pSP1 in which a DNA fragment carrying the lipase gene and the gene encoding the protein involved in the production thereof was inserted in between the HindIII- and SacI digestion sites of pMFY 42. Fig.6 shows the restriction map of pSP1. Herein, the white arrow and black arrow represent the same as in Fig. 1. "Km^r" represents kanamycin-resistant gene; and "Tc^r" represents tetracycline-resistant gene.

Two oligonucleotides, namely a commercially available M13 primer M4 (manufactured by TaKaRa Brewery, K. K.) and the oligonucleotide as shown as Sequence No.9, were mixed with the plasmid pS1S for polymerase chain reaction (PCR), to recover a DNA fragment containing only the lipase gene. The fragment was completely digested with the HindIII and SacI, and the resulting digested products were then linked to the plasmid pMFY42 in the same manner as described above, to recover a DNA fragment containing only the lipase gene in between the HindIII-and SacI digestion sites of pMFY 42. Fig.7 shows the restriction map of pSP2 (the symbols in the figure are the same as in Fig.6).

By electrophoresis, Pseudomonas sp. strain SD 705 (Accession No. FERM BP-4772) was transformed using the plasmids pSP1 and pSP2, to select kanamycin-resistant colonies. More specifically, firstly, the strain SD705 was grown in an L liquid medium (5 ml) adjusted to pH 9, until the OD reached 0.5. The bacteria were recovered by centrifugation. The bacteria were suspended in sterilized water, which were then again recovered and resuspend in sterilized water (0.5 ml). Adding the plasmid DNA to the bacterial suspension, which was then transferred into a cell with electrodes, a high-voltage electric pulse was applied to the DNA. Subsequently adding the L liquid medium (1 ml), pH 9 into the bacterial suspension, followed by shaking culture at 37°C for one hour, the resulting suspension was coated on an L plate medium, pH 9 containing 50 ppm kanamycin and olive oil emulsion as a lipase substrate. After overnight culturing at 35°C, grown colonies with clear zones formed around themselves were selected to recover a transformed strain.

Strain LD 9 as a lipase depletion strain of Pseudomonas mendocina strain SD 702 (Accession No. FERM BP-4291) was transformed to generate a transformant. By making N-methyl-N'-nitro-N-nitrosoguanidine act on Pseudomonas

Example 3

Preparation of oligonucleotide probe

The N-terminal amino acid sequence of the purified lipase was analyzed by a protein sequencer Model 476A (manufactured by Applied Biosystems, Co., Ltd.), to recover consequently the Sequence No.5. Based on the Sequence, an oligonucleotide probe as shown as Sequence No.6 was prepared on a DNA synthesizer. This probe was labeled, using ECL (trade name; 3'-oligolabelling and detection system, Amasham life science, Co., Ltd.).

Example 4

Isolation of gene encoding lipase

The transformant of about 1,000 colonies was overnight cultured on a nylon filter placed on an L plate medium at 37°C . Peeling off the filter, the bacteria were lyzed for 10 minutes on a filter impregnated with 0.5 M sodium hydroxide/1.5 M sodium chloride, which were then neutralized two times on a filter impregnated with 1.5 M sodium chloride/1 M Tris-HCl buffer, pH 7 for 7 minutes. After firing the filter at 80°C for 2 hours, the remaining bacteria were washed in 0.5% SDS/6 x SSC (1 x SSC means a 150 mM sodium chloride/15 mM sodium citrate solution; n x SSC means a solution at n-fold concentrations of 150 mM sodium chloride/15 mM sodium citrate solution). In a 0.1% SDS/5 x SSC/ 5 x Denhardts solution [0.1% phycol, 0.1% polyvinyl pyrrolidone, and 0.1% bovine serum albumin (BSA)], the pre-hybridization on the filter was carried out at 60°C for one hour. To an identical solution was added the labelled oligonucleotide probe for the hybridization on the filter overnight at 60°C . Subsequently, the filter was washed in 0.1% SDS/1 x SSC at 60°C for 15 minutes and then in 0.1% SDS/0.5 x SSC at 60°C for 15 minutes. This was subjected to detection according to the protocol of ECL (trade name; 3'-oligolabelling and detection system).

As the consequence of such colony hybridization, a number of positive colonies were recovered. From one of the resulting colonies, a plasmid was recovered, followed by digestion with restriction endonucleases EcoRI and PstI, SacI and XbaI, to prepare fragments. These fragments were separated by agarose electrophoresis to estimate the length of the inserted fragment, which indicates that a DNA fragment of about 7 kbp was recovered. The plasmid pS1 was digested with a variety of restriction endonucleases, to prepare a restriction map. Further, the plasmid was separated by agarose gel electrophoresis and adsorbed on a nylon filter. By the same procedures as for colony hybridization, southern hybridization was carried out. Finally, the plasmid was hybridized with a HindIII -SacI fragment of about 4 kbp and an EcoRI fragment of about 2.7 kbp. Consequently, it was assumed that the gene encoding lipase S and the gene encoding the protein involved in the production thereof was estimated to be carried in the HindIII -Sac I fragment of about 4 kbp and the EcoRI fragment of about 2.7 kbp, respectively. Fig.1 shows the restriction map of pS1. Herein, the white arrow depicts the lipase gene; the black arrow depicts the gene responsible for the lipase production; "Plac" represents lac promoter; "ori" represents replication initiating point; and "Ap^r" represents ampicillin-resistant gene. Recovering these fragments and individually linking the fragments to the HindIII-SacI site or EcoRI site of the plasmid pUC118 (manufactured by TaKaRa Brewery, K.K.), thereby transforming the Escherichia coli strain JM 101, plasmids pS1S and pS1E individually containing the respective DNA fragments were recovered. Figs.2 and 3 depict the restriction maps of pS1S and pS1E, respectively (the symbols in the figures are the same as in Fig.1).

Example 5

Determination of the nucleotide sequence of lipase gene

Using the plasmid pS1E, the nucleotide sequences of the gene encoding the lipase S and the gene encoding the protein involved in the production of the lipase S were determined according to the dideoxy method by Sanger (Sanger, F., Nicklen, S., Coulson, A.R. (1977), Proc. Natl. Acad. Sci. USA., 74, 5463). More specifically, the nucleotide sequencing was carried out on a DNA sequencer (Model 370A; Applied Biosystems, Co., Ltd.) using a dideoxy terminator sequencing kit (Applied Biosystems, Co. Ltd.). Consequently, the lipase-encoding nucleotide sequence of the Sequence No.2 and the nucleotide sequence encoding the protein involved in the production of the lipase, as shown as Sequence No.4, were recovered. The putative amino acid sequences based on these sequences are shown as Sequence Nos. 1 and 3.

generation and purification are not limited to the methods described above, but as a matter of course other methods are applicable.

Properties of lipase S

The lipase S thus produced by the methods described above has the following properties.

1. Action
The lipase S acts on triglyceride to hydrolyze its esters.
2. Substrate specificity
The lipase S hydrolyzes a wide variety of glycerides and esters.
3. Active pH and optimum pH
When measured in a range of pH 8 to 12 using the pH-stat method on a substrate olive oil, the active pH is 8 to 12 while the optimum pH is about 10.7.
4. Active temperature and optimum temperature
The active temperature and optimum temperature are 30 to 80°C and 60± 5 °C, respectively, when measured using triolein emulsion as the substrate within a range of 30 to 80°C.
5. Effects of detergents
The lipase S has a higher activity in solutions of various detergents containing protease.

Best Mode of Carrying out the Invention

The present invention will now be described with reference to the following examples, but the invention is not limited to these examples.

Example 1

Preparation of chromosomal DNA

Supplementing an L medium (1% polypeptone, 0.5% yeast extract, and 0.5% sodium chloride) with 10% sodium carbonate (3 ml) and adjusting the medium to pH 9, inoculating then the *Pseudomonas* sp. strain SD 705 in the resulting L medium (1000 ml), prior to overnight culturing at 35°C, and centrifuging the resulting medium, the bacteria were recovered. The bacteria were suspended in a 50 mM Tris-HCl buffer, pH 8 (8 ml) containing 0.4 M sodium chloride and 10 mM EDTA. Adding lysozyme and RNase A thereto so as to reach final concentrations of 0.5 mg/ml and 0.05 mg/ml, respectively, followed by gentle shaking at 37°C for 30 minutes, and further adding sodium dodecyl sulfate (SDS) to the resulting mixture so as to reach a final concentration of 1%, prior to gentle shaking at 37°C for 30 minutes, the bacteria were lyzed. Subsequently, the bacteria were heated at 60°C for 10 minutes, for complete solubilization. To the resulting solution was added an equal amount of phenol saturated with TE buffer (1 mM EDTA-containing 10 mM Tris-HCl buffer, pH 8), followed by gentle mixing while the vessel was inversely held with the bottom upward, and after centrifugation, the resulting upper aqueous phase was recovered. The above procedure was repeated three times. To the resulting aqueous phase recovered at the third time was added a 3-fold volume of ethanol cooled at - 20°C, to wind and isolate the precipitate around a plastic bar. The precipitate was then rinsed with ethanol, dried under reduced pressure and again solubilized in the TE buffer (1 ml).

Example 2

Preparation of chromosomal DNA library

After partially digesting the chromosomal DNA with a restriction endonuclease Sau3AaI, followed by agarose electrophoresis, a 2- to 10-kbp DNA fragment was recovered. Alternatively, a plasmid pUC 118 was digested with BamHI, for subsequent treatment with alkali protease. The two fragments were linked together with T4 DNA ligase. After culturing the resulting linking product in the L medium, the culture was then added to *Escherichia coli* strain JM 101 (0.3 ml) treated with 50 mM calcium chloride, followed by incubation at 0°C for 30 minutes. To the resulting mixture was added the L medium (to a final volume of 1 ml), followed by shaking at 37°C for one hour. The resulting culture was coated on an L plate medium containing 50 ppm ampicillin, for overnight culturing at 37°C. Consequently, a tranformant of about 1,000 colonies was recovered.

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(54) NOVEL LIPASE GENE AND PROCESS FOR THE PRODUCTION OF LIPASE WITH THE USE OF THE SAME

(57) A lipase gene isolated from the chromosomal DNA of *Pseudomonas* sp. SD705 strain (FERM BP-4772); a gene encoding a protein which participates in the production of a lipase; and a process for the production of the lipase with the use of these genes. The use of these genes makes it possible to efficiently produce a lipase which is industrially useful in detergents, food processing, paper making, oil manufacturing, etc.

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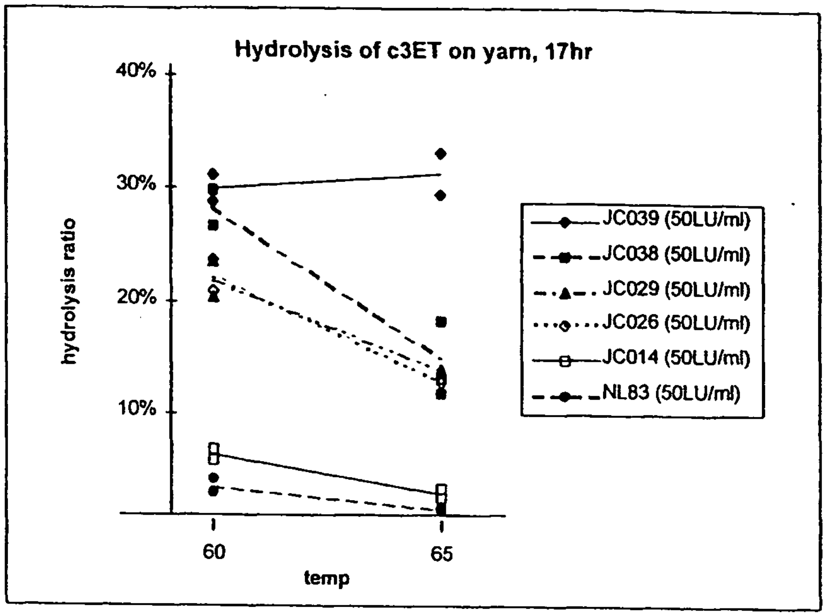


Fig. 4

Hydrolysis of c3ET on yarn, 17 hr

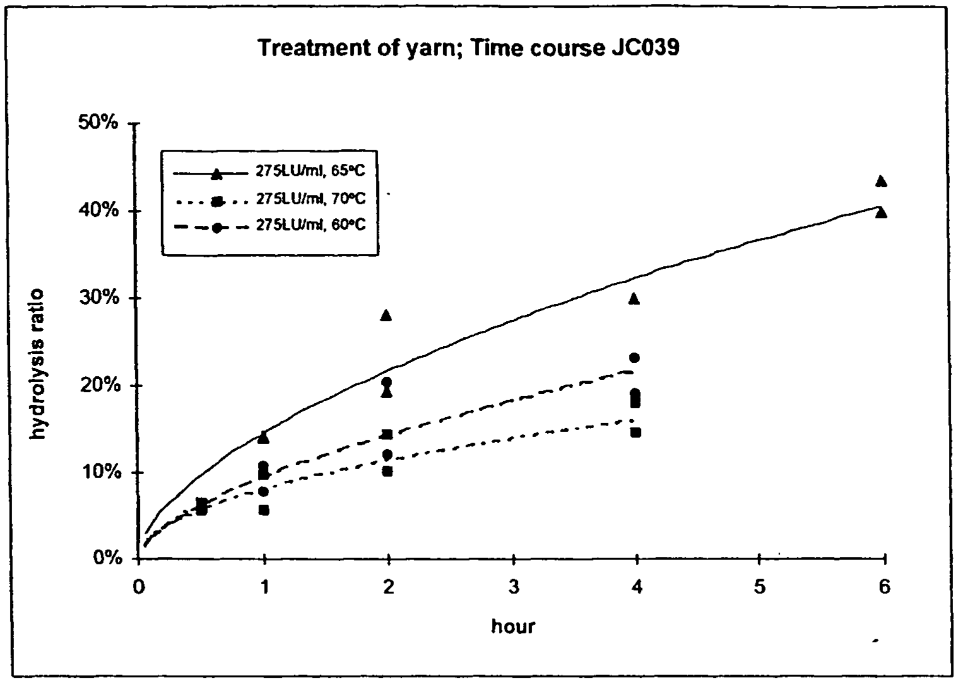


Fig. 5

Treatment of yarn; time course for JC039

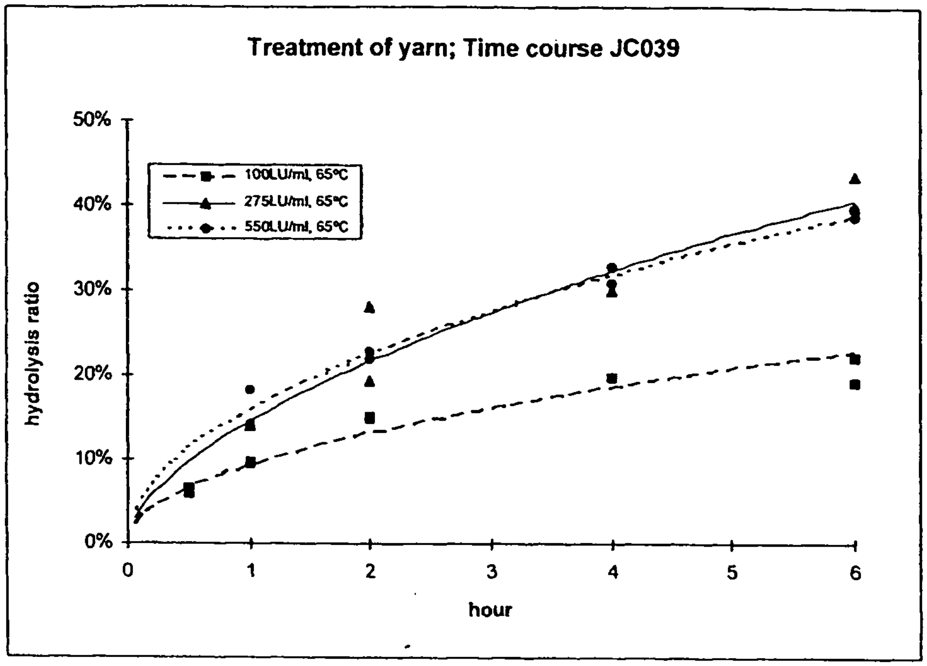


Fig. 6

Treatment of yarn; time course for JC039

(A) ORGANISM: Pseudomonas sp.
(B) STRAIN: SD 705

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:1..864

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
TTC GGC TCC TCG AAC TAC ACC AAG ACC CAG TAC CCG ATC GTC CTG ACC   48
Phe Gly Ser Ser Asn Tyr Thr Lys Thr Gln Tyr Pro Ile Val Leu Thr
1               5                   10                  15
CAC GGC ATG CTC GGT TTC GAC AGC CTG CTT GGA GTC GAC TAC TGG TAC   96
His Gly Met Leu Gly Phe Asp Ser Leu Leu Gly Val Asp Tyr Trp Tyr
            20                  25                  30
GGC ATT CCC TCA GCC CTG CGT AAA GAC GGC GCC ACC GTC TAC GTC ACC  144
Gly Ile Pro Ser Ala Leu Arg Lys Asp Gly Ala Thr Val Tyr Val Thr
        35                  40                  45
GAA GTC AGC CAG CTC GAC ACC TCC GAA GCC CGA GGT GAG CAA CTG CTG  192
Glu Val Ser Gln Leu Asp Thr Ser Glu Ala Arg Gly Glu Gln Leu Leu
    50                  55                  60
ACC CAA GTC GAG GAA ATC GTG GCC ATC AGC GGC AAG CCC AAG GTC AAC  240
Thr Gln Val Glu Glu Ile Val Ala Ile Ser Gly Lys Pro Lys Val Asn
65                  70                  75                  80
CTG TTC GGC CAC AGC CAT GGC GGG CCT ACC ATC CGC TAC GTT GCC GCC  288
Leu Phe Gly His Ser His Gly Gly Pro Thr Ile Arg Tyr Val Ala Ala
                85                  90                  95
GTG CGC CCG GAT CTG GTC GCC TCG GTC ACC AGC ATT GGC GCG CCG CAC  336
Val Arg Pro Asp Leu Val Ala Ser Val Thr Ser Ile Gly Ala Pro His
            100                 105                 110
AAG GGT TCG GCC ACC GCC GAC TTC ATC CGC CAG GTG CCG GAA GGA TCG  384
Lys Gly Ser Ala Thr Ala Asp Phe Ile Arg Gln Val Pro Glu Gly Ser
        115                 120                 125
GCC AGC GAA GCG ATT CTG GCC GGG ATC GTC AAT GGT CTG GGT GCG CTG  432
Ala Ser Glu Ala Ile Leu Ala Gly Ile Val Asn Gly Leu Gly Ala Leu
    130                 135                 140
ATC AAC TTC CTT TCC GGC AGC AGT TCG GAC ACC CCA CAG AAC TCG CTG  480
Ile Asn Phe Leu Ser Gly Ser Ser Ser Asp Thr Pro Gln Asn Ser Leu
145                 150                 155                 160
```

```
GGC ACG CTG GAG TCA CTG AAC TCC GAA GGC GCC GCA CGG TTT AAC GCC   528
Gly Thr Leu Glu Ser Leu Asn Ser Glu Gly Ala Ala Arg Phe Asn Ala
                165                 170                 175
CGC TTC CCC CAG GGG GTA CCA ACC AGC GCC TGC GGC GAG GGC GAT TAC   576
Arg Phe Pro Gln Gly Val Pro Thr Ser Ala Cys Gly Glu Gly Asp Tyr
            180                 185                 190
GTG GTC AkT GGC GTG CGC TAT TAC TCC TGG AGC GGC ACC ACC CCG CTG   624
Val Val Asn Gly Val Arg Tyr Tyr Ser Trp Ser Gly Thr Ser Pro Leu
        195                 200                 205
ACC AAC GTA CTC GAC CCC TCC GAC CTG CTG CTC GGC GCC ACC TCC CTG   672
Thr Asn Val Leu Asp Pro Ser Asp Leu Leu Leu Gly Ala Thr Ser Leu
    210                 215                 220
ACC TTC GGT TTC GAG GCC AAC GAT GGT CTG GTC GGA CGC TGC AGC TCC   720
Thr Phe Gly Phe Glu Ala Asn Asp Gly Leu Val Gly Arg Cys Ser Ser
225                 230                 235                 240
CGG CTG GGT ATG GTG ATC CGC GAC AAC TAC CGG ATG AAC CAC CTG GAC   768
Arg Leu Gly Met Val Ile Arg Asp Asn Tyr Arg Met Asn His Leu Asp
                245                 250                 255
GAG GTG AAC CAG ACC TTC GGG CTG ACC AGC ATC TTC GAG ACC AGC CCG   816
Glu Val Asn Gln Thr Phe Gly Leu Thr Ser Ile Phe Glu Thr Ser Pro
            260                 265                 270
GTA TCG GTC TAT CGC CAG CAA GCC AAT CGC CTG AAG AAC GCC GGG CTC   864
Val Ser Val Tyr Arg Gln Gln Ala Asn Arg Leu Lys Asn Ala Gly Leu
        275                 280                 285
```

(2) INFORMATION FOR SEQ ID NO: 3

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 335 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
- (A) ORGANISM: Pseudomonas sp.
- (B) STRAIN: SD 705

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
Met Lys Pro Leu Ile Tyr Leu Pro Leu Leu Leu Gly Leu Gly Leu Leu
1               5                   10                  15
Gly Trp His Leu Ser Thr Pro Ala Pro Ser Pro Ser Ser Ala Ser Pro
            20                  25                  30
Ala Pro Pro Gln Val Ser Ser Glu Lys Pro Ala Thr Ala His Met Asp
        35                  40                  45
Leu Thr Arg Pro Val Ala Arg Ser Thr Asp Gln His Leu Pro Ala Ser
    50                  55                  60
Leu Arg Asp Thr Asp Val Asp Gly Gln Leu Glu Val Asp Ala Gln Gly
65                  70                  75                  80
Asn Leu Val Ile Ser Asp Gln Leu Arg His Leu Phe Asp Tyr Phe Phe
                85                  90                  95
Ser Thr Val Gly Glu Gln Ser Phe Glu Gln Ala Ser Thr Gly Ile Arg
            100                 105                 110
Asp Tyr Leu Ala Ser Gln Leu Arg Glu Pro Ala Leu Gly Gln Ala Leu
        115                 120                 125
Asp Leu Leu Asp Arg Tyr Ile Asn Tyr Lys Thr Glu Leu Val Glu Leu
    130                 135                 140
Glu Arg Arg Phe Pro Met Val Thr Glu Leu Asp Gly Leu Arg Ala Arg
    145                 150                 155                 160
Glu Asp Ala Val Gln Arg Leu Arg Ala Ser Leu Phe Asn Ala Gln Glu
                165                 170                 175
His Ala Ala Phe Phe Ala Ser Glu Glu Val Tyr Asn Gln Phe Thr Leu
            180                 185                 190
Glu Arg Leu Ala Ile Leu His Asp Pro Ser Leu Asp Pro Gln Asp Lys
        195                 200                 205
Ala Glu Arg Ile Glu Arg Leu Arg Glu Gly Leu Pro Asp Glu Leu Gln
    210                 215                 220
Gln Leu Leu Val Pro Gln Leu His Leu Thr Leu Arg Gln Gln Thr Gln
225                 230                 235                 240
Gln Leu Leu Glu Gln Gly Ala Glu Pro Glu Gln Leu Arg Gln Leu Arg
                245                 250                 255
Leu Asn Leu Val Gly Pro Gln Ala Thr Glu Arg Leu Glu Ala Leu Asp
            260                 265                 270
Arg Gln Arg Ser Glu Trp Asp Gln Arg Leu Ser Gly Phe Asn Arg Glu
        275                 280                 285
Arg Gln Ala Ile Ile Ser Gln Pro Gly Leu Ala Asp Ser Asp Lys Gln
    290                 295                 300
```

Ala Ala Ile Glu Ala Leu Leu His Glu Gln Phe Ser Glu His Glu Arg
305 310 315 320
Leu Arg Val Ser Ser Leu Leu Gly Leu Asp Ser Arg Ala Glu Arg
325 330 335

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1005 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Pseudomonas sp.
(B) STRAIN: SD 705

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:1..1005

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ATG AAG CCG CTG ATT TAT CTG CCT TTG CTT CTT GGC CTG GGG CTG CTC 48
Met Lys Pro Leu Ile Tyr Leu Pro Leu Leu Leu Gly Leu Gly Leu Leu
1 5 10 15
GGC TGG CAC CTG AGC ACG CCG GCA CCC AGC CCA TCC AGC GCC TCA CCA 96
Gly Trp His Leu Ser Thr Pro Ala Pro Ser Pro Ser Ser Ala Ser Pro
20 25 30
GCG CCG CCA CAA GTC AGC AGT GAA AAA CCT GCC ACG GCT CAC ATG GAC 144
Ala Pro Pro Gln Val Ser Ser Glu Lys Pro Ala Thr Ala His Met Asp
35 40 45
CTG ACC CGC CCG GTG GCC CGC AGC ACC GAC CAG CAT CTG CCC GCC TCG 192
Leu Thr Arg Pro Val Ala Arg Ser Thr Asp Gln His Leu Pro Ala Ser
50 55 60
CTG CGC GAT ACC GAC GTC GAT GGC CAG CTG GAG GTC GAC GCC CAG GGC 240
Leu Arg Asp Thr Asp Val Asp Gly Gln Leu Glu Val Asp Ala Gln Gly
65 70 75 80
AAT CTG GTG ATT TCC GAC CAG CTG CGC CAC CTG TTC GAC TAT TTC TTC 288
Asn Leu Val Ile Ser Asp Gln Leu Arg His Leu Phe Asp Tyr Phe Phe
85 90 95

```
AGC ACC GTC GGC GAA CAG TCG TTC GAG CAG GCC AGC ACC GGT ATC CGC   336
Ser Thr Val Gly Glu Gln Ser Phe Glu Gln Ala Ser Thr Gly Ile Arg
            100                 105                 110
GAC TAT CTG GCC AGC CAG CTG CGT GAA CCG GCT CTG GGT CAG GCC CTG   384
Asp Tyr Leu Ala Ser Gln Leu Arg Glu Pro Ala Leu Gly Gln Ala Leu
        115                 120                 125
GAT CTG CTG GAT CGC TAT ATC AAC TAC AAG ACC GAG CTG GTG GAA CTG   432
Asp Leu Leu Asp Arg Tyr Ile Asn Tyr Lys Thr Glu Leu Val Glu Leu
    130                 135                 140
GAG CGA CGC TTC CCG ATG GTG ACC GAG CTG GAC GGC CTG CGT GCC CGT   480
Glu Arg Arg Phe Pro Met Val Thr Glu Leu Asp Gly Leu Arg Ala Arg
145                 150                 155                 160
GAA GAT GCC GTA CAG CGC CTG CGC GCC AGC CTG TTC AAC GCG CAG GAG   528
Glu Asp Ala Val Gln Arg Leu Arg Ala Ser Leu Phe Asn Ala Gln Glu
                165                 170                 175
CAC GCC GCC TTC TTC GCC AGC GAA GAG GTC TAT AAC CAG TTC ACT CTT   576
His Ala Ala Phe Phe Ala Ser Glu Glu Val Tyr Asn Gln Phe Thr Leu
            180                 185                 190
GAG CGT CTG GCG ATA CTG CAC GAC CCG TCG CTG GAT CCG CAG GAC AAG   624
Glu Arg Leu Ala Ile Leu His Asp Pro Ser Leu Asp Pro Gln Asp Lys
        195                 200                 205
GCC GAG CGG ATC GAA CGT CTG CGC GAA GGG CTA CCC GAC GAG TTG CAA   672
Ala Glu Arg Ile Glu Arg Leu Arg Glu Gly Leu Pro Asp Glu Leu Gln
    210                 215                 220
CAA TTG CTG GTA CCG CAA TTA CAC CTG ACC CTG CGC CAG CAG ACC CAG   720
Gln Leu Leu Val Pro Gln Leu His Leu Thr Leu Arg Gln Gln Thr Gln
225                 230                 235                 240
CAG TTG CTG GAG CAA GGC GCC GAG CCG GAA CAG CTA CGC CAA TTG CGC   768
Gln Leu Leu Glu Gln Gly Ala Glu Pro Glu Gln Leu Arg Gln Leu Arg
                245                 250                 255
CTG AAC CTG GTC GGC CCC CAG GCA ACC GAA CGC CTG GAG GCA CTG GAC   816
Leu Asn Leu Val Gly Pro Gln Ala Thr Glu Arg Leu Glu Ala Leu Asp
            260                 265                 270
CGC CAG CGC AGC GAA TGG GAT CAG CGC CTG AGC GGC TTC AAT CGC GAA   864
Arg Gln Arg Ser Glu Trp Asp Gln Arg Leu Ser Gly Phe Asn Arg Glu
        275                 280                 285
```

```
CGG CAG GCG ATC ATC AGC CAG CCG GGG CTG GCC GAC AGT GAC AAG CAG   912
Arg Gln Ala Ile Ile Ser Gln Pro Gly Leu Ala Asp Ser Asp Lys Gln
    290                 295                 300
GCC GCG ATT GAG GCC CTG CTG CAC GAG CAG TTC AGT GAG CAT GAG CGG   960
Ala Ala Ile Glu Ala Leu Leu His Glu Gln Phe Ser Glu His Glu Arg
305                 310                 315                 320
CTG AGG GTC AGC AGT CTG CTG GGA CTC GAT AGC CGC GCC GAA CGC       1005
Leu Arg Val Ser Ser Leu Leu Gly Leu Asp Ser Arg Ala Glu Arg
                325                 330                 335
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Pseudomonas sp.
(B) STRAIN: SD 705

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Phe Gly Ser Ser Asn Tyr Thr Lys Thr Gln Tyr Pro Ile Val Leu Thr
1               5                   10                  15
His Gly Met Leu Gly Phe Asp Ser Leu Leu
            20                  25
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 nucleic acids
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
AACTACACNA AGACNCAGTA      20
```

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleic acids
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGGGAATTCA GGACTCGCAT TATGCGCAAC 30

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleic acids
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TTTGAATTCA GAGCCCGGCG TTCTTCAGGC 30

2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleic acids
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TTTGAGCTCA GAGCCCGGCG TTCTTCAGGC 30

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleic acids
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AAATCTAGAT TCGGCTCCTC GAACTACACC 30

2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleic acids
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCCTCTAGAC TAGCGTTCGG CGCGGCTATC 30

2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleic acids
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CCCTCTAGAT CAGAGCCCGG CGTTCTTCAG 30

2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleic acids
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CCCGAATTCA TACGAATTAA AGTTGAAAGC 30

2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleic acids
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

AAATCTAGAG TTGAAACCAA TTAAGTACTC 30

2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleic acids
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GGGTCTAGAT CCCTAAGGAT GTACTGGATG 30

2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleic acids
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid, synthetic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TTTAAGCTTA GAAACTCAAC TGTCACAGTG 30

Claims

1. A gene encoding the amino acid sequence of Sequence No.1.

2. A gene carrying the lipase-encoding nucleotide sequence of Sequence No.2.

3. A gene encoding the amino acid sequence of Sequence No.3.

4. A gene carrying the nucleotide sequence encoding the protein involved in the production of lipase, as described as Sequence No.4.

5. A gene from bacteria of genus Pseudomonas according to any one of claims 1 to 4.

6. A gene from Pseudomonas sp. strain SD 705 (FERM BP-4772), according to any one of claims 1 to 4.

7. A DNA containing the whole or a part of the nucleotide sequence of the gene according to any one of claims 1 to 6.

8. A DNA hybridizable with the whole or a part of the nucleotide sequence of the gene according to claim 3 or 4.

9. A recombinant DNA generated by incorporating at least one of the genes according to claims 1 to 4 into a replicable vector in a host microbial cell for the expression of the genes.

10. A recombinant chromosomal DNA generated by the homologous incorporation of at least one of the genes according to claims 1 to 4 in a microbial chromosome.

11. A transformed host microorganism, having been transformed with the recombinant DNA according to claim 9.

12. A transformed microorganism containing the recombinant chromosomal DNA according to claim 10.

13. A transformed microorganism according to claim 11, wherein the microorganism is a bacterium of genus Escherichia, a bacterium of genus Pseudomonas, or a bacterium of genus Bacillus.

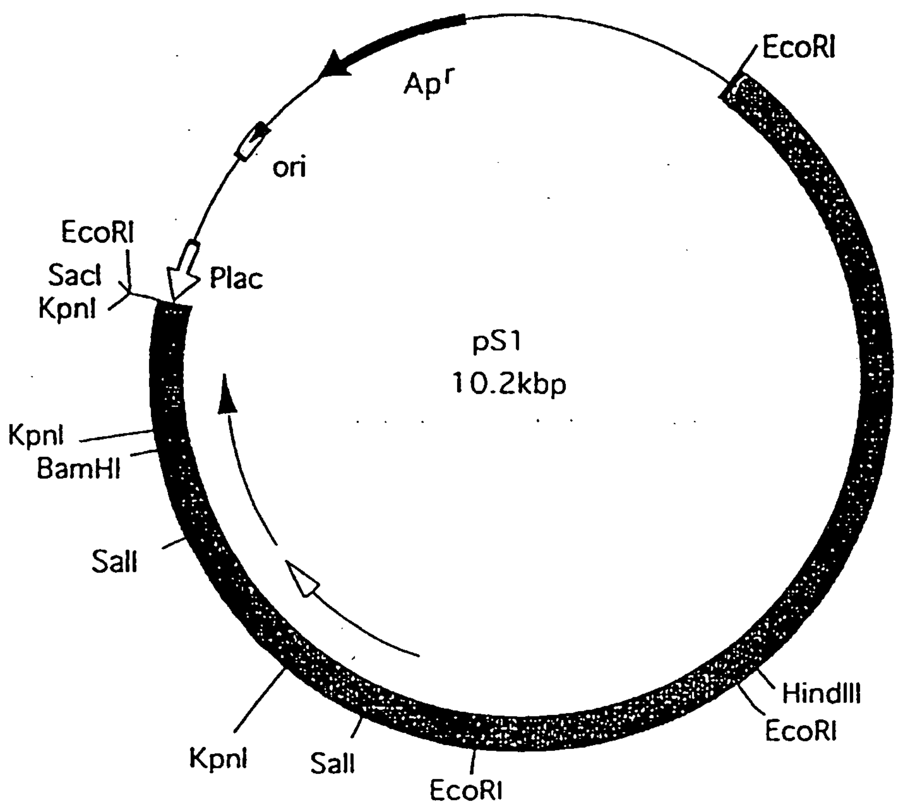
14. A transformed microorganism according to claim 12, wherein the microorganism is a bacterium of genus Pseudomonas or a bacterium of genus Bacillus.

15. A transformed microorganism according to claim 11 or 12, wherein the microorganism is Pseudomonas alcaligenes, Pseudomonas pseudoalcaligenes, Pseudomonas mendocina, or Bacillus subutilus.

16. A transformed microorganism according to claim 11 or 12, wherein the microorganism is Pseudomonas sp. strain SD 705, Pseudomonas alcaligenes strain SD 702, Bacillus strain NKS-21 or a variant thereof.

17. A method for producing a lipase, comprising culturing at least one of the transformed host cells according to claims 11 to 16 to produce a culture containing a lipase and isolating the lipase.

FIG. 1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ori
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KpnI
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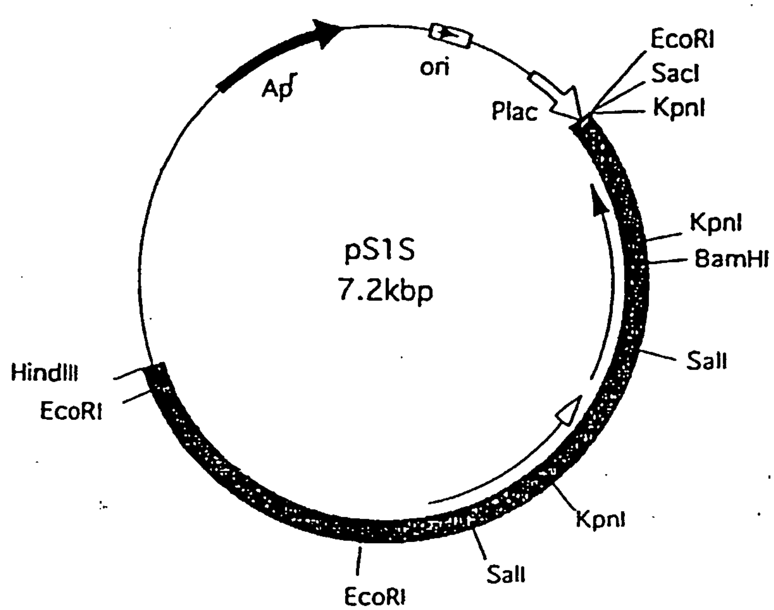
SalI
KpnI
SalI
EcoRI
HindIII
EcoRI

FIG. 2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SacI
KpnI
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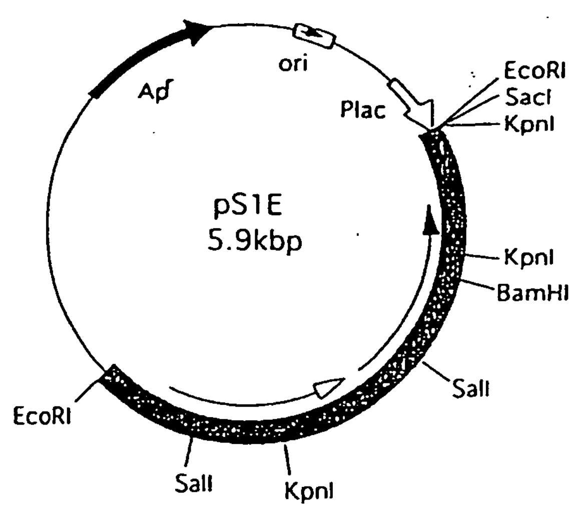
KpnI
BamHI
SalI
HindIII
EcoRI
KpnI
SalI
EcoRI

FIG. 3


ori
Apr
Plac
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KpnI
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SalI
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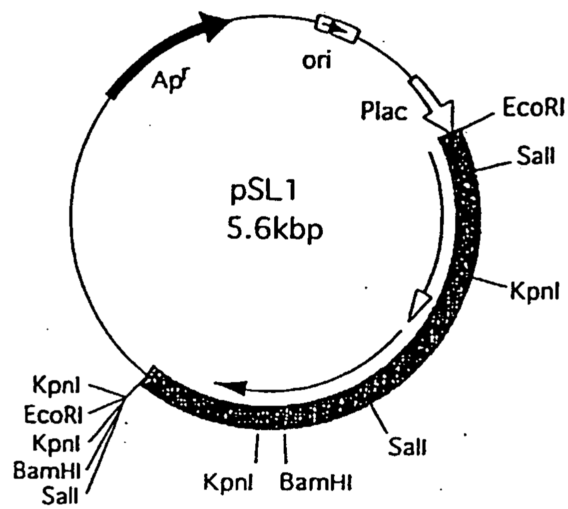
KpnI

FIG. 4


Ap^r
ori
Plac
EcoRI
SalI
pSL1
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KpnI
KpnI
EcoRI
KpnI
BamHI
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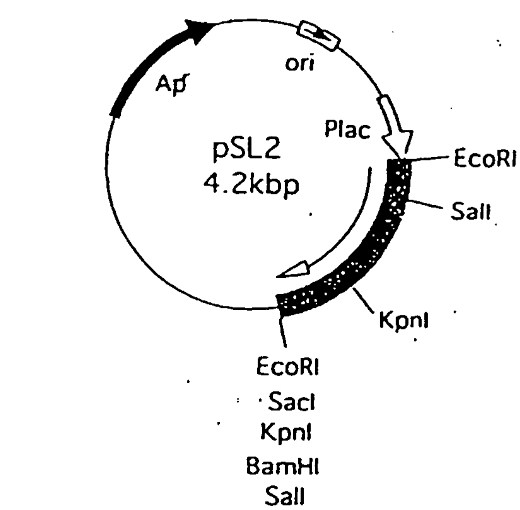
BamHI
SalI

FIG. 5


Ap^r
ori
Plac
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SacI
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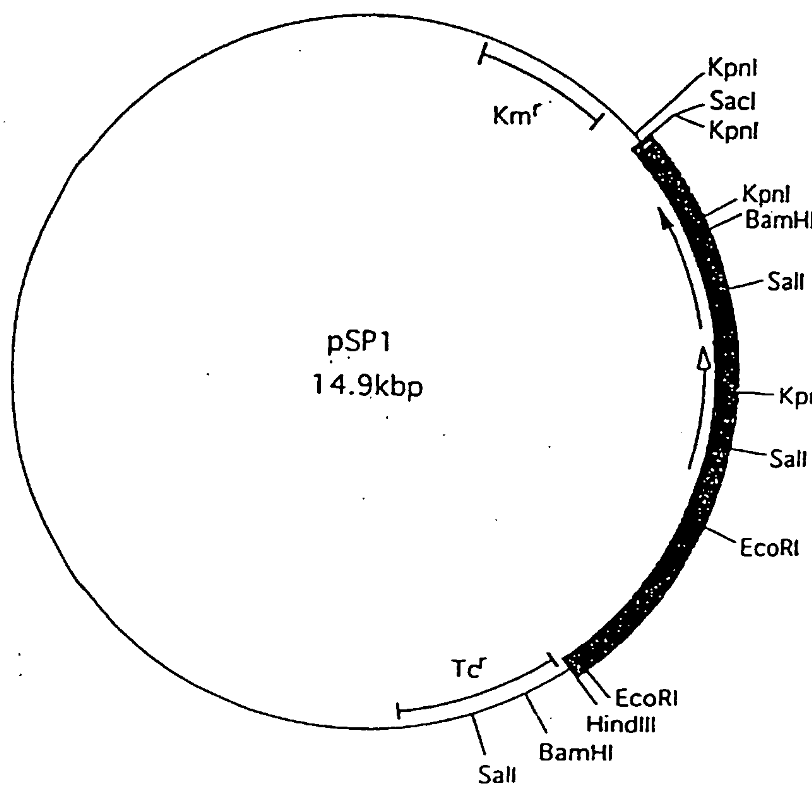
BamHI
SalI

FIG. 6


KpnI
SacI
KpnI
Km^r
KpnI
BamHI
SalI
pSP1
14.9kbp
KpnI
SalI
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Tc^r
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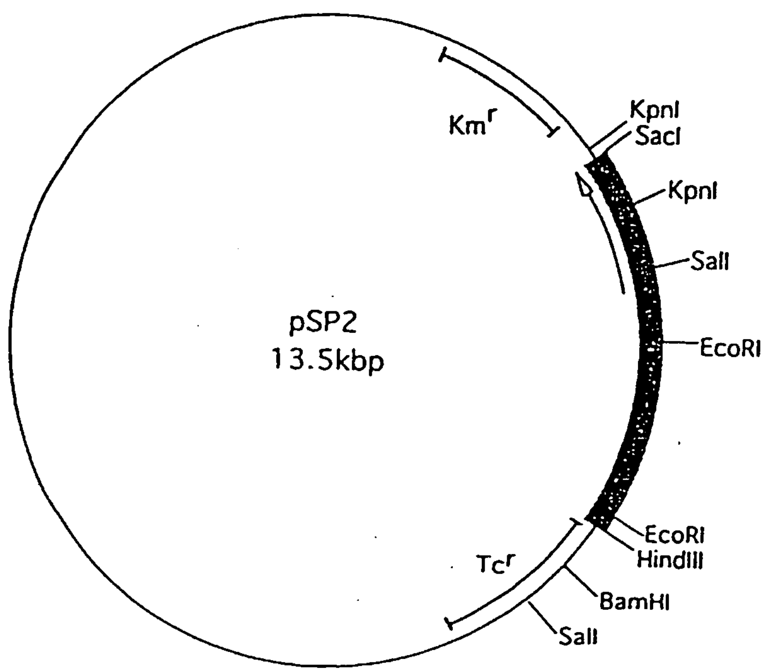
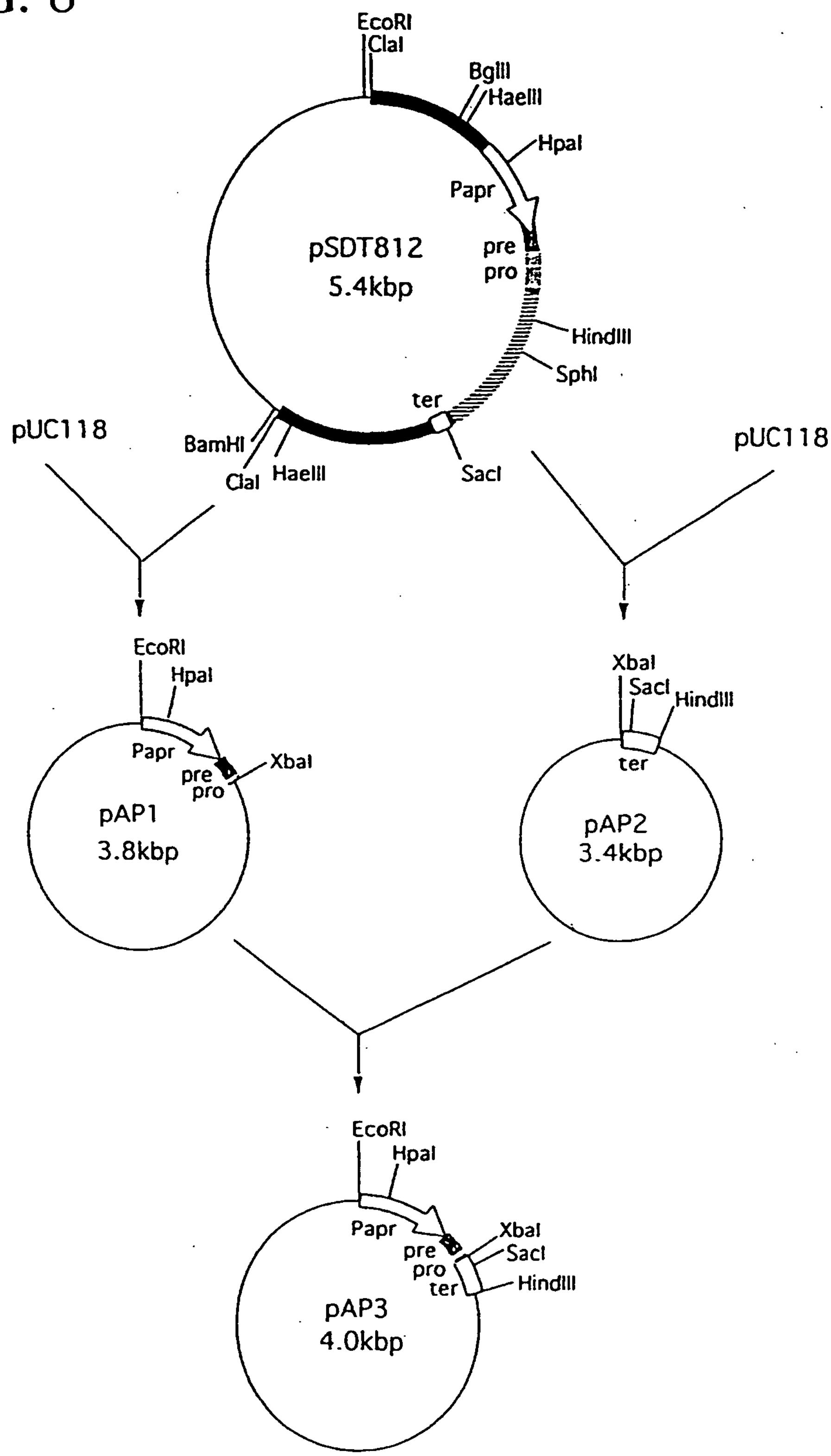
HindIII
BamHI
SalI

FIG. 7



Km^r
KpnI
SacI
KpnI
SalI
EcoRI
pSP2
13.5kbp
EcoRI
HindIII
Tc^r
BamHI
SalI

FIG. 8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ClaI
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HaeIII
HpaI
Papr
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5.4kbp
pre
pro
HindIII
SphI
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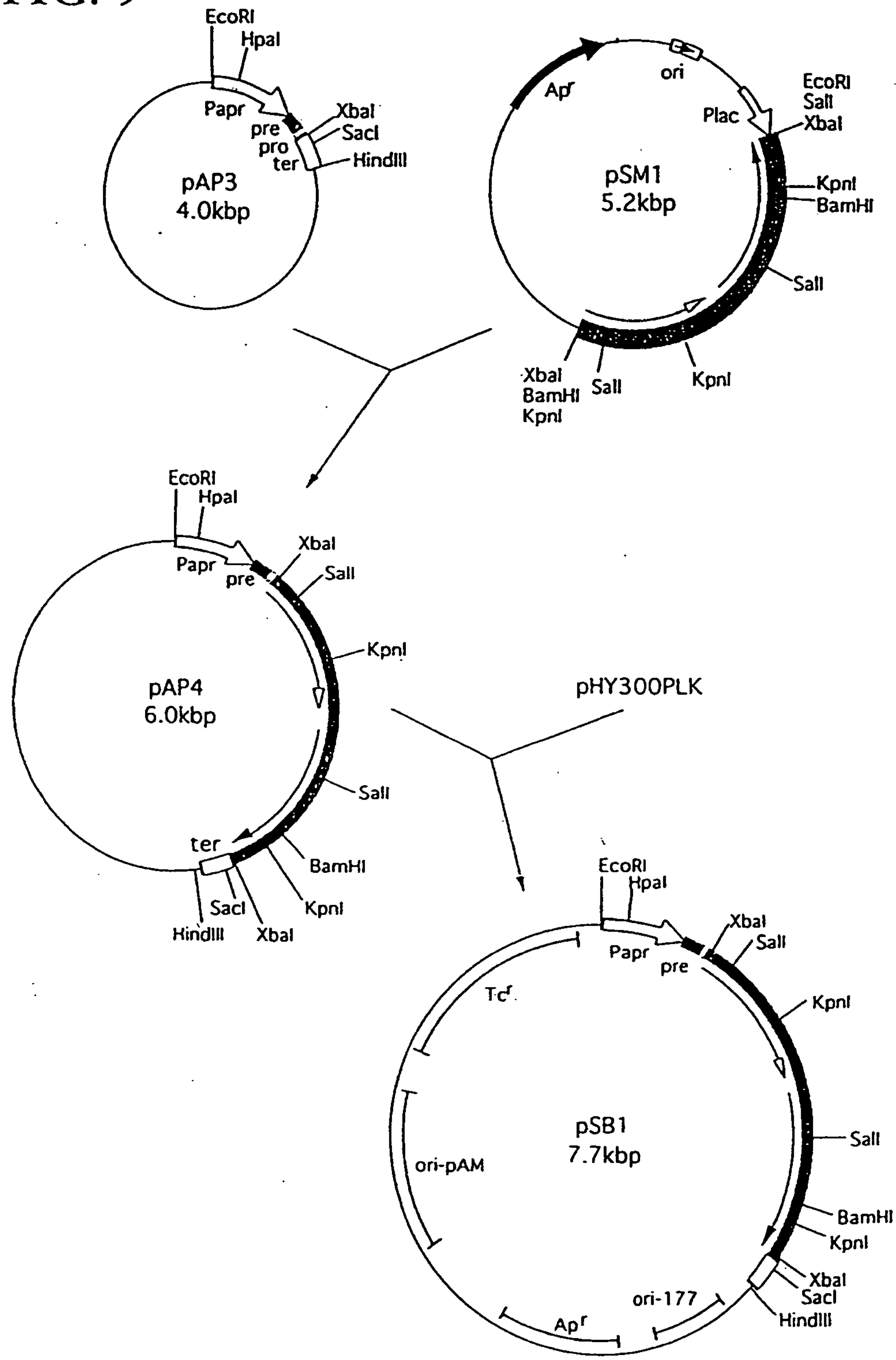
BamHI
ClaI
HaeIII
SacI
pUC118
EcoRI
HpaI
Papr
pre
pro
XbaI
pAP1
3.8kbp
XbaI
SacI
HindIII
ter
pAP2
3.4kbp
EcoRI
HpaI
Papr
pre
pro
ter
XbaI
SacI
HindIII
pAP3
4.0kbp

FIG. 9


EcoRI
HpaI
Papr
pre
pro
ter
XbaI
SacI
HindIII
pAP3
4.0kbp
Apr
ori
Plac
EcoRI
SalI
XbaI
pSM1
5.2kbp
KpnI
BamHI
SalI
XbaI
BamHI
KpnI
SalI
KpnI
EcoRI
HpaI
Papr
pre
XbaI
SalI
KpnI
pAP4
6.0kbp
SalI
ter
BamHI
KpnI
SacI
HindIII
XbaI
pHY300PLK
EcoRI
HpaI
Papr
pre
XbaI
SalI
Tcr
KpnI
pSB1
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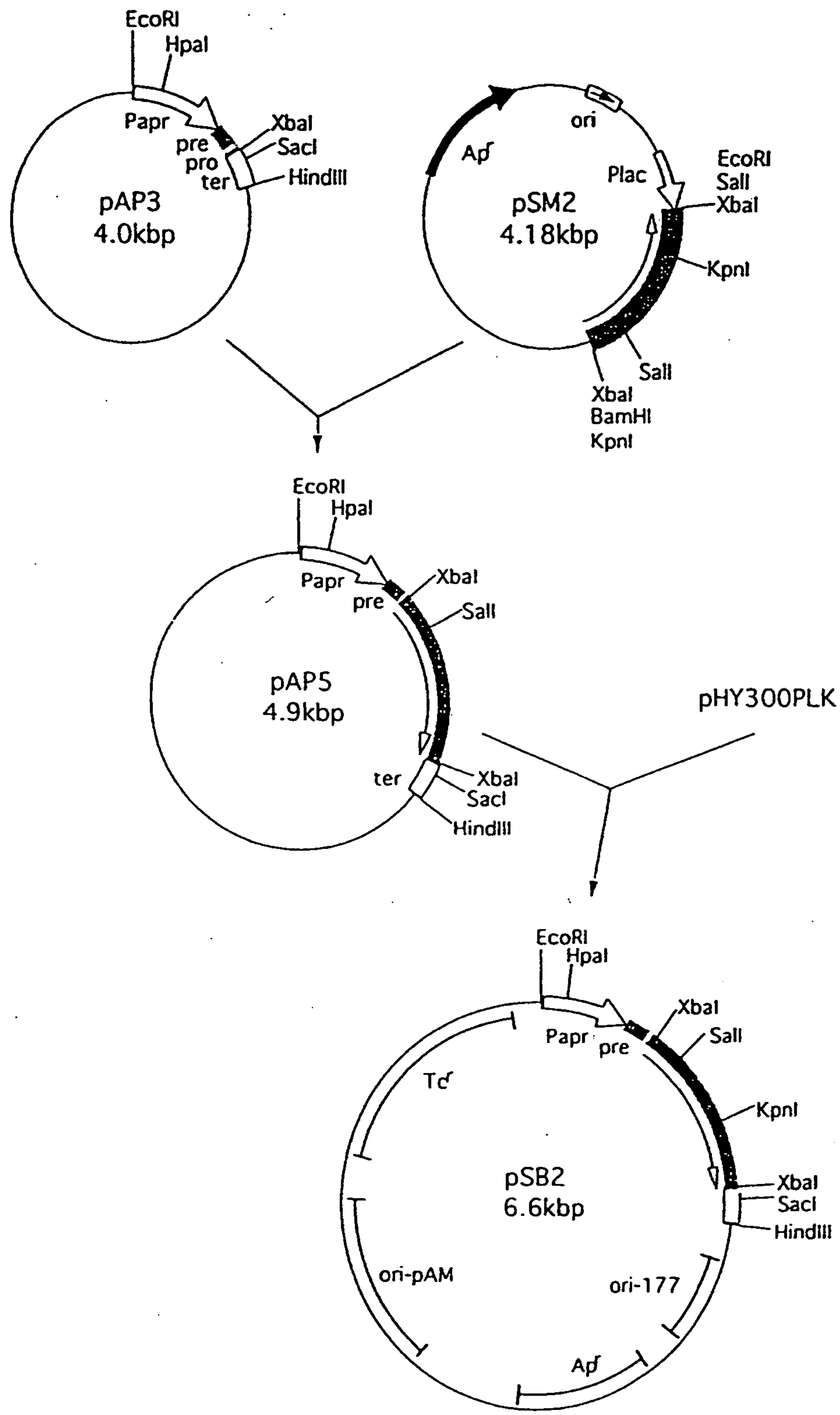
ori-pAM
SalI
BamHI
KpnI
XbaI
SacI
HindIII
ori-177
Apr

FIG. 10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HpaI
Papr
pre
pro
ter
XbaI
SacI
HindIII
pAP3
4.0kbp
Apr
ori
Plac
EcoRI
SalI
XbaI
pSM2
4.18kbp
KpnI
SalI
XbaI
BamHI
KpnI
EcoRI
HpaI
Papr
pre
XbaI
SalI
pAP5
4.9kbp
ter
XbaI
SacI
HindIII
pHY300PLK
EcoRI
HpaI
XbaI
SalI
Papr
pre
Tcr
KpnI
pSB2
6.6kbp
XbaI
SacI
HindIII
ori-pAM
ori-177
Apr

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP96/00426

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl[6] C12N9/20, 15/55 // (C12N9/20, C12R1:38), (C12N15/55, C12R1:38)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl[6] C12N9/20, 15/55

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, BIOSIS PREVIEWS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GBF Monogr., 16 (Lipases) (1991), pp. 417-20	1 - 17
A	Journal of Biochemistry (Tokyo), Vol. 112, No. 5 (1992), pp. 598-603	1 - 17
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☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
May 15, 1996 (15. 05. 96)	May 28, 1996 (28. 05. 96)
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